

(19)



Europäisches Patentamt

European Patent Office

Office européen des brevets



(11)

**EP 0 872 554 A2**

(12)

**EUROPEAN PATENT APPLICATION**

(43) Date of publication:

21.10.1998 Bulletin 1998/43

(51) Int. Cl.<sup>6</sup>: **C12N 15/52**, C12P 23/00,

A23K 1/16, A23L 1/275,

C12N 1/21

(21) Application number: **97120324.5**

(22) Date of filing: **20.11.1997**

(84) Designated Contracting States:

**AT BE CH DE DK ES FI FR GB GR IE IT LI LU MC  
NL PT SE**

Designated Extension States:

**AL LT LV MK RO SI**

(30) Priority: **02.12.1996 EP 96810839**

(71) Applicant:

**F. HOFFMANN-LA ROCHE AG  
4070 Basel (CH)**

• **Tsygankov, Yuri,**

**State Scient. Cnt. of Russian Fed  
Moscow 113545 (RU)**

(74) Representative: **Braun, Axel et al**

**F.Hoffmann-La Roche AG  
Patent Department (PLP),  
124 Grenzacherstrasse  
4070 Basel (CH)**

Remarks:

The applicant has subsequently filed a sequence listing and declared, that it includes no new matter.

(72) Inventors:

• **Pasamontes, Luis  
4632 Trimbach (CH)**

**(54) Improved fermentative carotenoid production**

(57) The present invention is directed to processes for the preparation of canthaxanthin, adonixanthin, astaxanthin, a mixture of adonixanthin and astaxanthin and zeaxanthin by a cell which has been transformed by DNA sequences encoding the respective biosynthetic enzymes of Flavobacterium and the gram negative bacterium E-396. Furthermore the present invention is directed to a food or feed composition comprising one or more of the aforementioned carotenoids.

**EP 0 872 554 A2**

## Description

Over 600 different carotenoids have been described from carotenogenic organisms found among bacteria, yeast, fungi and plants. Currently only two of them,  $\beta$ -carotene and astaxanthin are commercially produced in microorganisms and used in the food and feed industry.  $\beta$ -carotene is obtained from algae and astaxanthin is produced in *Pfaffia* strains which have been generated by classical mutation. However, fermentation in *Pfaffia* has the disadvantage of long fermentation cycles and recovery from algae is cumbersome. Therefore it is desirable to develop production systems which have better industrial applicability, e.g. can be manipulated for increased titers and/or reduced fermentation times. Two such systems using the biosynthetic genes from *Erwinia herbicola* and *Erwinia uredovora* have already been described in WO 91/13078 and EP 393 690, respectively. Furthermore, three  $\beta$ -carotene ketolase genes ( $\beta$ -carotene  $\beta$ -4-oxygenase) of the marine bacteria *Agrobacterium aurantiacum* and *Alcaligenes* strain PC-1 (crtW) [Misawa, 1995, Biochem. Biophys. Res. Com. 209, 867-876][Misawa, 1995, J. Bacteriology 177, 6575-6584] and from the green algae *Haematococcus pluvialis* (bkt) [Lotan, 1995, FEBS Letters 364, 125-128][Kajiwar, 1995, Plant Mol. Biol. 29, 343-352] have been cloned. *E. coli* carrying either the carotenogenic genes (crtE, crtB, crtY and crtI) of *E. herbicola* [Hundle, 1994, MGG 245, 406-416] or of *E. uredovora* and complemented with the crtW gene of *A. aurantiacum* [Misawa, 1995] or the bkt gene of *H. pluvialis* [Lotan, 1995][Kajiwar, 1995] resulted in the accumulation of canthaxanthin ( $\beta$ , $\beta$ -carotene-4,4'-dione), originating from the conversion of  $\beta$ -carotene, via the intermediate echinenone ( $\beta$ , $\beta$ -carotene-4-one). Introduction of the above mentioned genes (crtW or bkt) into *E. coli* cells harbouring besides the carotenoid biosynthesis genes mentioned above also the crtZ gene of *E. uredovora* [Kajiwar, 1995][Misawa, 1995], resulted in both cases in the accumulation of astaxanthin (3,3'-dihydroxy- $\beta$ , $\beta$ -carotene-4,4'-dione). The results obtained with the bkt gene, are in contrast to the observation made by others [Lotan, 1995], who using the same experimental set-up, but introducing the *H. pluvialis* bkt gene in a zeaxanthin ( $\beta$ , $\beta$ -carotene-3,3'-diol) synthesising *E. coli* host harbouring the carotenoid biosynthesis genes of *E. herbicola*, a close relative of the above mentioned *E. uredovora* strain, did not observe astaxanthin production.

Since there is a continuing need in even more optimized fermentation systems for industrial application it is therefore in the first instance an object of the present invention to provide a process for the preparation of canthaxanthin by culturing under suitable culture conditions a cell which is transformed by a DNA sequence comprising the following DNA sequences:

- a) a DNA sequence which encodes the GGPP synthase of *Flavobacterium* sp. R1534 (crtE) or a DNA sequence which is substantially homologous;
  - b) a DNA sequence which encodes the prephytoene synthase of *Flavobacterium* sp. R1534 (crtB) or a DNA sequence which is substantially homologous;
  - c) a DNA sequence which encodes the phytoene desaturase of *Flavobacterium* sp. R1534 (crtI) or a DNA sequence which is substantially homologous;
  - d) a DNA sequence which encodes the lycopene cyclase of *Flavobacterium* sp. R1534 (crtY) or a DNA sequence which is substantially homologous;
  - e) a DNA sequence which encodes the  $\beta$ -carotene  $\beta$ 4-oxygenase of the microorganism E-396 (FERM BP-4283) [crtW<sub>E396</sub>] or a DNA sequence which is substantially homologous;
- or a cell which is transformed by a vector comprising DNA sequences specified above under a) to e) and by isolating canthaxanthin from such cells or the culture medium by methods known in the art.

Furthermore it is in the second instance an object of the present invention to provide a process for the preparation of a mixture of adonixanthin and astaxanthin or adonixanthin or astaxanthin alone by a process as mentioned above characterized therein that in addition to the DNA sequences specified under a) to e) the following additional DNA sequence is present:

- f) a DNA sequence which encodes the  $\beta$ -carotene hydroxylase of the microorganism E-396 (FERM BP-4283) [crtZ<sub>E396</sub>] or a DNA sequence which is substantially homologous;

and the DNA sequence specified under e) is as specified above or the following sequence:

- g) a DNA sequence which encodes the  $\beta$ -carotene  $\beta$ 4-oxygenase of *Alcaligenes* strain PC-1 (crtW) or a DNA

sequence which is substantially homologous;

and isolating the desired mixture of adonixanthin and astaxanthin or adonixanthin or a astaxanthin alone from such cells of the culture medium and separating the desired mixture or carotenoids alone from other carotenoids which might be present by methods known in the art.

Furthermore it is an object of the present invention to provide a process for the preparation of zeaxanthin by a process as claimed in the first instance characterized therein that the DNA sequence as specified under e) is replaced by the DNA sequence as specified under f) in the second instance and by isolating zeaxanthin from the cell or the culture medium and separating it from other carotenoids which might be present by methods known in the art.

Furthermore it is an object of the present invention to provide a process for the production of adonixanthin by culturing under suitable culture conditions a cell which is transformed by a DNA sequence comprising the following heterologous DNA sequences:

a) a DNA sequence which encodes the GGPP synthase of the microorganism E-396 (FERM BP-4283) [crtE<sub>E396</sub>] or a DNA sequence which is substantially homologous;

b) a DNA sequence which encodes the prephytoene synthase the microorganism E-396 (FERM BP-4283) [crtB<sub>E396</sub>] or a DNA sequence which is substantially homologous;

c) a DNA sequence which encodes the phytoene desaturase of the microorganism E-396 (FERM BP-4283) [crtI<sub>E396</sub>] or a DNA sequence which is substantially homologous;

d) a DNA sequence which encodes the lycopene cyclase of the microorganism E-396 (FERM BP-4283) [crtY<sub>E396</sub>] or a DNA sequence which is substantially homologous;

e) a DNA sequence which encodes the  $\beta$ -carotene hydroxylase of the microorganism E396 (FERM BP-4283) [crtZ<sub>E396</sub>] or a DNA sequence which is substantially homologous; and

f) a DNA sequence which encodes the  $\beta$ -carotene  $\beta$ 4-oxygenase of the microorganism E396 (FERM BP-4283) [crtW<sub>E396</sub>] or a DNA sequence which is substantially homologous;

and isolating adonixanthin from the cell or the culture medium and separating it from other carotenoids which might be present by methods known in the art.

Further it is an object of the present invention to provide a process as described above characterized therein that the transformed host cell is a prokaryotic host cell, like E. coli, Bacillus or Flavobacter and a process as described above characterized therein that the transformed host cell is a eukaryotic host cell, like yeast or a fungal cell.

Furthermore it is an object of the present invention to provide a DNA sequence comprising one or more DNA sequences selected from the group consisting of:

a) a DNA sequence which encodes the GGPP synthase of Flavobacterium sp. R1534 (crtE) or a DNA sequence which is substantially homologous;

b) a DNA sequence which encodes the prephytoene synthase of Flavobacterium sp. R1534 (crtB) or a DNA sequence which is substantially homologous;

c) a DNA sequence which encodes the phytoene desaturase of Flavobacterium sp. R1534 (crtI) or a DNA sequence which is substantially homologous;

d) a DNA sequence which encodes the lycopene cyclase of Flavobacterium sp. R1534 (crtY) or a DNA sequence which is substantially homologous; and

e) a DNA sequence which encodes the  $\beta$ -carotene hydroxylase of Flavobacterium sp. R1534 (crtZ) or a DNA sequence which is substantially homologous.

It is also an object of the present invention to provide a vector comprising such DNA sequence, preferably in the form of an expression vector. Furthermore it is an object of the present invention to provide a cell which is transformed

by such DNA sequence or vector, preferably which is a prokaryotic cell and more preferably which is *E. coli* or a *Bacillus* strain. Such transformed cell which is an eukaryotic cell, preferably a yeast cell or a fungal cell, is also an object of the present invention. Finally the present invention concerns also a process for the preparation of a desired carotenoid or a mixture of carotenoids by culturing such a cell under suitable culture conditions and isolating the desired carotenoid or a mixture of carotenoids from such cells or the culture medium and, in case only one carotenoid is desired separating it by methods known in the art from other carotenoids which might also be present and a process for the preparation of a food or feed composition characterized therein that after such a process has been effected the carotenoid or carotenoid mixture is added to food or feed.

Furthermore, a DNA sequence comprising the following DNA sequences is an object of the present invention:

a) a DNA sequence which encodes the GGPP synthase of *Flavobacterium* sp. R1534 (*crtE*) or a DNA sequence which is substantially homologous;

b) a DNA sequence which encodes the prephytoene synthase of *Flavobacterium* sp. R1534 (*crtB*) or a DNA sequence which is substantially homologous; and

c) a DNA sequence which encodes the phytoene desaturase of *Flavobacterium* sp. R 1534 (*crtI*) or a DNA sequence which is substantially homologous.

It is also an object of the present invention to provide a vector comprising such DNA sequence, preferably in the form of an expression vector. Furthermore it is an object of the present invention to provide a cell which is transformed by such DNA sequence or vector, preferably which is a prokaryotic cell and more preferably which is *E. coli* or a *Bacillus* strain. Such transformed cell which is an eukaryotic cell, preferably a yeast cell or a fungal cell, is also an object of the present invention. Finally the present invention concerns also a process for the preparation of a desired carotenoid or a mixture of carotenoids by culturing such a cell under suitable conditions and isolating the desired carotenoid or a mixture of carotenoids from such cells or the culture medium and, in case only one carotenoid is desired separating it by methods known in the art from other carotenoids which might also be present, preferably such a process for the preparation of lycopene and a process for the preparation of a food or feed composition characterized therein that after such a process has been effected the carotenoid, preferably lycopene or carotenoid mixture, preferably a lycopene comprising mixture is added to food or feed.

Furthermore a DNA sequence comprising the following DNA sequence is also an object of the present invention:

a) a DNA sequence which encodes the GGPP synthase of *Flavobacterium* sp. R1534 (*crtE*) or a DNA sequence which is substantially homologous;

b) a DNA sequence which encodes the prephytoene synthase of *Flavobacterium* sp. R1534 (*crtB*) or a DNA sequence which is substantially homologous;

c) a DNA sequence which encodes the phytoene desaturase of *Flavobacterium* sp. R1534 (*crtI*) or a DNA sequence which is substantially homologous; and

d) a DNA sequence which encodes the lycopene cyclase of *Flavobacterium* sp. R1534 (*crtY*) or a DNA sequence which is substantially homologous.

It is also an object of the present invention to provide a vector comprising such DNA sequence, preferably in the form of an expression vector. Furthermore it is an object of the present invention to provide a cell which is transformed by such DNA sequence or vector, preferably which is a prokaryotic cell and more preferably which is *E. coli* or a *Bacillus* strain. Such transformed cell which is an eukaryotic cell, preferably a yeast cell or a fungal cell, is also an object of the present invention. Finally the present invention concerns also a process for the preparation of a desired carotenoid or a mixture of carotenoids by culturing such a cell under suitable conditions and isolating the desired carotenoid or a mixture of carotenoids from such cells or the culture medium and, in case only one carotenoid is desired separating it by methods known in the art from other carotenoids which might also be present, preferably such a process for the preparation of  $\beta$ -carotene and a process for the preparation of a food or feed composition characterized therein that after such a process has been effected the carotenoid, preferably  $\beta$ -carotene or carotenoid mixture, preferably a  $\beta$ -carotene comprising mixture is added to food or feed.

Furthermore a cell which is transformed by the above mentioned DNA sequence comprising subsequences a) to d) or the vector comprising it and a second DNA sequence which encodes the  $\beta$ -carotene  $\beta$ 4-oxygenase of *Alcaligenes* strain PC-1 (*crt W*) or a DNA sequence which is substantially homologous or a second vector which comprises a DNA

sequence which encodes the  $\beta$ -carotene  $\beta$ 4-oxygenase of *Alcaligenes* strain PC-1 (crt W) or a DNA sequence which is substantially homologous; and a process for the preparation of a desired carotenoid or a mixture of carotenoids by culturing such cell under suitable conditions and isolating the desired carotenoid or a mixture of carotenoids from such cells or the culture medium and, in case only one carotenoid is desired separating it by methods known in the art from other carotenoids which might also be present, preferably such a process for the preparation of echinenone and a process for the preparation of a food or feed composition characterized therein that after such a process has been effected the carotenoid, preferably echinenone or carotenoid mixture, preferably an echinenone comprising mixture is added to food or feed.

Furthermore it is an object of the present invention to provide a DNA sequence as mentioned above comprising subsequences a) to d) and a DNA sequence which encodes the  $\beta$ -carotene  $\beta$ 4-oxygenase of *Alcaligenes* strain PC-1 (crt W) or a DNA sequence which is substantially homologous and a vector comprising such DNA sequence, preferably in the form of an expression vector. Furthermore it is an object of the present invention to provide a cell which is transformed by such DNA sequence or vector, preferably which is a prokaryotic cell and more preferably which is *E. coli* or a *Bacillus* strain. Such transformed cell which is an eukaryotic cell, preferably a yeast cell or a fungal cell, is also an object of the present invention. Finally the present invention concerns also a process for the preparation of a desired carotenoid or a mixture of carotenoids by culturing such a cell under suitable culture conditions and isolating the desired carotenoid or a mixture of carotenoids from such cells of the culture medium and, in case only one carotenoid is desired separating it by methods known in the art from other carotenoids which might also be present, especially such a process for the preparation of echinenone or canthaxanthin and a process for the preparation of a food or feed compositing characterized therein that after such a process has been effected the carotenoid, preferably echinenone or canthaxanthin or carotenoid mixture, preferably a echinenone or canthaxanthin containing mixture is added to food or feed.

Furthermore a DNA sequence comprising the following DNA sequences is also an object of the present invention:

a) a DNA sequence which encodes the GGPP synthase of *Flavobacterium* sp. R1534 (crtE) or a DNA sequence which is substantially homologous;

b) a DNA sequence which encodes the prephytoene synthase of *Flavobacterium* sp. R1534 (crtB) or a DNA sequence which is substantially homologous;

c) a DNA sequence which encodes the phytoene desaturase of *Flavobacterium* sp. R1534 (crtI) or a DNA sequence which is substantially homologous;

d) a DNA sequence which encodes the lycopene cyclase of *Flavobacterium* sp. R1534 (crtY) or a DNA sequence which is substantially homologous; and

e) a DNA sequence which encodes the  $\beta$ -carotene hydroxylase of *Flavobacterium* sp. R1534 (crtZ) or a DNA sequence which is substantially homologous.

It is also an object of the present invention to provide a vector comprising such DNA sequence, preferably in the form of an expression vector. Furthermore it is an object of the present invention to provide a cell which is transformed by such DNA sequence or vector, preferably which is a prokaryotic cell and more preferably which is *E. coli* or a *Bacillus* strain. Such transformed cell which is an eukaryotic cell, preferably a yeast cell or a fungal cell, is also an object of the present invention. Finally the present invention concerns also a process for the preparation of a desired carotenoid or mixture of carotenoids by culturing such a cell under suitable conditions and isolating the desired carotenoid or a mixture of carotenoids from such cells of the culture medium and, in case only one carotenoid is desired separating it by methods known in the art from other carotenoids which might also be present, preferably such a process for the preparation of zeaxanthin and a process for the preparation of a food or feed composition characterized therein that after such a process has been effected the carotenoid, preferably zeaxanthin or the carotenoid mixture, preferably a zeaxanthin containing mixture is added to food or feed.

Furthermore a DNA sequence as mentioned above comprising subsequences a) to e) and in addition a DNA sequence which encodes the  $\beta$ -carotene  $\beta$ 4-oxygenase of *Alcaligenes* strain PC-1 (crt W) or a DNA sequence which is substantially homologous is an object of the present invention and to provide a vector comprising such DNA sequence, preferably in form of an expression vector. Furthermore it is an object of the present invention to provide a cell which is transformed by such DNA sequence or vector, preferably which is a prokaryotic cell and more preferably which is *E. coli* or a *Bacillus* strain. Such transformed cell which is an eukaryotic cell, preferably a yeast cell or a fungal cell, is also an object of the present invention. Finally the present invention concerns also a process for the preparation of a desired carotenoid or a mixture of carotenoids by culturing such a cell under suitable culture conditions and isolating the desired carotenoid or a mixture of carotenoids from such cells of the culture medium and, in case only one carotenoid is desired

separating it by methods known in the art from other carotenoids which might also be present, preferably such a process for the preparation of zeaxanthin, adonixanthin or astaxanthin and a process for the preparation of a food or feed composition characterized therein that after such a process has been effected the carotenoid, preferably zeaxanthin, adonixanthin or astaxanthin or carotenoid mixture, preferably a zeaxanthin, adonixanthin or astaxanthin containing mixture is added to food or feed.

Furthermore a cell which is transformed by the DNA sequence mentioned above comprising subsequences a) to e) or a vector comprising such DNA sequence and a second DNA sequence which encodes the  $\beta$ -carotene  $\beta$ 4-oxygenase of *Alcaligenes* strain PC-1 (crt W) or a DNA sequence which is substantially homologous or a second vector which comprises a DNA sequence which encodes the  $\beta$ -carotene  $\beta$ 4-oxygenase of *Alcaligenes* strain PC-1 (crt W) or a DNA sequence which is substantially homologous is also an object of the present invention and a process for the preparation of a desired carotenoid or a mixture of carotenoids by culturing such a cell under suitable conditions and isolating the desired carotenoid or a mixture of carotenoids from such cells or the culture medium, and in case only one carotenoid is desired separating it by methods known in the art from other carotenoids which might also be present, preferably such a process for the preparation of zeaxanthin or adonixanthin and a process for the preparation of a food or feed composition characterized therein that after such a process has been effected the carotenoid, preferably zeaxanthin or adonixanthin or carotenoid mixture, preferably a zeaxanthin or adonixanthin containing mixture is added to food or feed.

Furthermore it is an object of the present invention to provide the DNA sequences and vectors as specified before and a process for the preparation of a food or feed composition characterized therein that after a process as specified before has been effected the carotenoid prepared by such process is added to food or feed.

In this context it should be mentioned that the expression "a DNA sequence is substantially homologous" refers with respect to the crtE encoding DNA sequence to a DNA sequence which encodes an amino acid sequence which shows more than 45 %, preferably more than 60 % and more preferably more than 75 % and most preferably more than 90 % identical amino acids when compared to the amino acid sequence of crtE of *Flavobacterium* sp. 1534 and is the amino acid sequence of a polypeptide which shows the same type of enzymatic activity as the enzyme encoded by crtE of *Flavobacterium* sp. 1534. In analogy with respect to crtB this means more than 60 %, preferably more than 70 %, more preferably more than 80 % and most preferably more than 90 %; with respect to crtI this means more than 70 %, preferably more than 80 % and most preferably more than 90 %; with respect to crtY this means 55 %, preferably 70 %, more preferably 80 % and most preferably 90 %.

"DNA sequences which are substantially homologous" refer with respect to the crtW<sub>E396</sub> encoding DNA sequence to a DNA sequence which encodes an amino acid sequence which shows more than 60%, preferably more than 75% and most preferably more than 90% identical amino acids when compared to the amino acid sequence of crtW of the microorganism E 396 (FERM BP-4283) and is the amino acid sequence of a polypeptide which shows the same type of enzymatic activity as the enzyme encoded by crtW of the microorganism E 396. In analogy with respect to crtZ<sub>E396</sub> this means more than 75%, preferable more than 80% and most preferably more than 90%; with respect to crtE<sub>E396</sub>, crtB<sub>E396</sub>, crtI<sub>E396</sub>, crtY<sub>E396</sub> and crtZ<sub>E396</sub> this means more than 80%, preferably more than 90% and most preferably 95%.

DNA sequences in form of genomic DNA, cDNA or synthetic DNA can be prepared as known in the art [see e.g. Sambrook et al., Molecular Cloning, Cold Spring Harbor Laboratory Press 1989] or, e.g. as specifically described in Examples 1, 2 or 7. In the context of the present invention it should be noted that all DNA sequences used for the process for production of carotenoids of the present invention encoding crt-gene products can also be prepared as synthetic DNA sequences according to known methods or in analogy to the method specifically described for crtW in Example 7.

The cloning of the DNA-sequences of the present invention from such genomic DNA can then be effected, e.g. by using the well known polymerase chain reaction (PCR) method. The principles of this method are outlined e.g. in PCR Protocols: A guide to Methods and Applications, Academic Press, Inc. (1990). PCR is an in vitro method for producing large amounts of a specific DNA of defined length and sequence from a mixture of different DNA-sequences. Thereby, PCR is based on the enzymatic amplification of the specific DNA fragment of interest which is flanked by two oligonucleotide primers which are specific for this sequence and which hybridize to the opposite strand of the target sequence. The primers are oriented with their 3' ends pointing toward each other. Repeated cycles of heat denaturation of the template, annealing of the primers to their complementary sequences and extension of the annealed primers with a DNA polymerase result in the amplification of the segment between the PCR primers. Since the extension product of each primer can serve as a template for the other, each cycle essentially doubles the amount of the DNA fragment produced in the previous cycle. By utilizing the thermostable Taq DNA polymerase, isolated from the thermophilic bacteria *Thermus aquaticus*, it has been possible to avoid denaturation of the polymerase which necessitated the addition of enzyme after each heat denaturation step. This development has led to the automation of PCR by a variety of simple temperature-cycling devices. In addition, the specificity of the amplification reaction is increased by allowing the use of higher temperatures for primer annealing and extension. The increased specificity improves the overall yield of amplified products by minimizing the competition by non-target fragments for enzyme and primers. In this way the specific sequence of interest is highly amplified and can be easily separated from the non-specific sequences by methods known in the

art, e.g. by separation on an agarose gel and cloned by methods known in the art using vectors as described e.g. by Holten and Graham in *Nucleic Acid Res.* 19, 1156 (1991), Kovalic et. al. in *Nucleic Acid Res.* 19, 4560 (1991), Marchuk et al. in *Nucleic Acid Res.* 19, 1154 (1991) or Mead et al. in *Bio/Technology* 9, 657-663 (1991).

The oligonucleotide primers used in the PCR procedure can be prepared as known in the art and described e.g. in Sambrook et al., s.a.

Amplified DNA-sequences can then be used to screen DNA libraries by methods known in the art (Sambrook et al., s.a.) or as specifically described in Examples 1 and 2.

Once complete DNA-sequences of the present invention have been obtained they can be used as a guideline to define new PCR primers for the cloning of substantially homologous DNA sequences from other sources. In addition they and such homologous DNA sequences can be integrated into vectors by methods known in the art and described e.g. in Sambrook et al. (s.a.) to express or overexpress the encoded polypeptide(s) in appropriate host systems. However, a man skilled in the art knows that also the DNA-sequences themselves can be used to transform the suitable host systems of the invention to get overexpression of the encoded polypeptide. Appropriate host systems are for example Bacteria e.g. *E. coli*, Bacilli as, e.g. *Bacillus subtilis* or *Flavobacter* strains. *E. coli*, which could be used are *E. coli* K12 strains e.g. M15 [described as DZ 291 by Villarejo et al. in *J. Bacteriol.* 120, 466-474 (1974)], HB 101 [ATCC No. 33694] or *E. coli* SG13009 [Gottesman et al., *J. Bacteriol.* 148, 265-273 (1981)]. Suitable *Flavobacter* strains can be obtained from any of the culture collections known to the man skilled in the art and listed, e.g. in the journal "Industrial Property" (January 1994, pgs 29-40), like the American Type Culture Collection (ATCC) or the Centraalbureau voor Schimmelkultures (CBS) and are, e.g. *Flavobacterium* sp. R 1534 (ATCC No. 21588, classified as unknown bacterium; or as CBS 519.67) or all *Flavobacter* strains listed as CBS 517.67 to CBS 521.67 and CBS 523.67 to CBS 525.67, especially R 1533 (which is CBS 523.67 or ATCC 21081, classified as unknown bacterium; see also USP 3,841,967). Further *Flavobacter* strains are also described in WO 91/03571. Suitable eukaryotic host systems are for example fungi, like *Aspergilli* e.g. *Aspergillus niger* [ATCC 9142] or yeasts, like *Saccharomyces*, e.g. *Saccharomyces cerevisiae* or *Pichia*, like *pastoris*, all available from ATCC.

Suitable vectors which can be used for expression in *E. coli* are mentioned, e.g. by Sambrook et al. [s.a.] or by Fiers et al. in *Proc. 8th Int. Biotechnology Symposium* [Soc. Franc. de Microbiol., Paris (Durand et al., eds.), pp. 680-697 (1988)] or by Bujard et al. in *Methods in Enzymology*, eds. Wu and Grossmann, Academic Press, Inc. Vol. 155, 416-433 (1987) and Stüber et al. in *Immunological Methods*, eds. Lefkovits and Pernis, Academic Press, Inc., Vol. IV, 121-152 (1990). Vectors which could be used for expression in Bacilli are known in the art and described, e.g. in EP 405 370, EP 635 572 *Proc. Nat. Acad. Sci. USA* 81, 439 (1984) by Yansura and Henner, *Meth. Enzym.* 185, 199-228 (1990) or EP 207 459. Vectors which can be used for expression in fungi are known in the art and described e.g. in EP 420 358 and for yeast in EP 183 070, EP 183 071, EP 248 227, EP 263 311. Vectors which can be used for expression in *Flavobacter* are known in the art and described in the Examples or, e.g. in *Plasmid Technology*, ed. by J. Grinstead and P.M. Bennett, Academic Press (1990).

Once such DNA-sequences have been expressed in an appropriate host cell in a suitable medium the carotenoids can be isolated either from the medium in the case they are secreted into the medium or from the host organism and, if necessary separated from other carotenoids if present in case one specific carotenoid is desired by methods known in the art (see e.g. *Carotenoids Vol IA: Isolation and Analysis*, G. Britton, S. Liaaen-Jensen, H. Pfander; 1995, Birkhäuser Verlag, Basel).

The carotenoids of the present invention can be used in a process for the preparation of food or feeds. A man skilled in the art is familiar with such process. Such compound foods or feeds can further comprise additives or components generally used for such purpose and known in the state of the art.

After the invention has been described in general hereinbefore, the following figures and examples are intended to illustrate details of the invention, without thereby limiting it in any matter.

**Figure 1:** The biosynthesis pathway for the formation of carotenoids of *Flavobacterium* sp. R1534 is illustrated explaining the enzymatic activities which are encoded by DNA sequences of the present invention.

**Figure 2:** Southern blot of genomic *Flavobacterium* sp. R1534 DNA digested with the restriction enzymes shown on top of each lane and hybridized with Probe 46F. The arrow indicated the isolated 2.4 kb *XhoI/PstI* fragment.

**Figure 3:** Southern blot of genomic *Flavobacterium* sp. R1534 DNA digested with *Clal* or double digested with *Clal* and *HindIII*. Blots shown in Panel A and B were hybridized to probe A or probe B, respectively (see examples). Both *Clal/HindIII* fragments of 1.8 kb and 9.2 kb are indicated.

**Figure 4:** Southern blot of genomic *Flavobacterium* sp. R1534 DNA digested with the restriction enzymes shown on top of each lane and hybridized to probe C. The isolated 2.8 kb *Sa1I/HindIII* fragment is shown by the

arrow.

- 5 **Figure 5:** Southern blot of genomic *Flavobacterium* sp. R1534 DNA digested with the restriction enzymes shown on top of each lane and hybridized to probe D. The isolated BclI/SphI fragment of approx. 3 kb is shown by the arrow.
- 10 **Figure 6:** Physical map of the organization of the carotenoid biosynthesis cluster in *Flavobacterium* sp. R1534, deduced from the genomic clones obtained. The location of the probes used for the screening are shown as bars on the respective clones.
- 15 **Figure 7:** Nucleotide sequence of the *Flavobacterium* sp. R1534 carotenoid biosynthesis cluster and its flanking regions. The nucleotide sequence is numbered from the first nucleotide shown (see BamHI site of Fig. 6). The deduced amino acid sequence of the ORF's (orf-5, orf-1, crtE, crtB, crtI, crtY, crtZ and orf-16) are shown with the single-letter amino acid code. Arrow (-->) indicate the direction of the transcription; asterisks, stop codons.
- 20 **Figure 8:** Protein sequence of the GGPP synthase (crtE) of *Flavobacterium* sp. R1534 with a MW of 31331 Da.
- 25 **Figure 9:** Protein sequence of the prephytoene synthetase (crtB) of *Flavobacterium* sp. R1534 with a MW of 32615 Da.
- Figure 10:** Protein sequence of the phytoene desaturase (crtI) of *Flavobacterium* sp. R1534 with a MW of 54411 Da.
- 30 **Figure 11:** Protein sequence of the lycopene cyclase (crtY) of *Flavobacterium* sp. R1534 with a MW of 42368 Da.
- Figure 12:** Protein sequence of the  $\beta$ -carotene hydroxylase (crtZ) of *Flavobacterium* sp. R1534 with a MW of 19282 Da.
- 35 **Figure 13:** Recombinant plasmids containing deletions of the *Flavobacterium* sp. R1534 carotenoid biosynthesis gene cluster.
- 40 **Figure 14:** Primers used for PCR reactions. The underlined sequence is the recognition site of the indicated restriction enzyme. Small caps indicate nucleotides introduced by mutagenesis. Boxes show the artificial RBS which is recognized in *B. subtilis*. Small caps in bold show the location of the original adenine creating the translation start site (ATG) of the following gene (see original operon). All the ATG's of the original *Flavobacter* carotenoid biosynthetic genes had to be destroyed to not interfere with the rebuild transcription start site. Arrows indicate start and ends of the indicated *Flavobacterium* R1534 WT carotenoid genes.
- 45 **Figure 15:** Linkers used for the different constructions. The underlined sequence is the recognition site of the indicated restriction enzyme. Small caps indicate nucleotides introduced by synthetic primers. Boxes show the artificial RBS which is recognized in *B. subtilis*. Arrow indicate start and ends of the indicated *Flavobacterium* carotenoid genes.
- Figure 16:** Construction of plasmids pBIKS(+)-clone59-2, pLyco and pZea4.
- Figure 17:** Construction of plasmid p602CAR.
- 50 **Figure 18:** Construction of plasmids pBIKS(+)-CARVEG-E and p602 CARVEG-E.
- Figure 19:** Construction of plasmids pHP13-2CARZYIB-EINV and pHP13-2PN25ZYIB-EINV.
- Figure 20:** Construction of plasmid pXI12-ZYIB-EINVMUTRBS2C.
- 55 **Figure 21:** Northern blot analysis of *B. subtilis* strain BS1012::ZYIB-EINV4. Panel A: Schematic representation of a reciprocal integration of plasmid pXI12-ZYIB-EINV4 into the levan-sucrase gene of *B. subtilis*. Panel B: Northern blot obtained with probe A (PCR fragment which was obtained with CAR 51 and CAR 76 and

hybridizes to the 3' end of crtZ and the 5' end of crtY). Panel C: Northern blot obtained with probe B (BamHI-XhoI fragment isolated from plasmid pBIIKS(+)-crtE/2 and hybridizing to the 5' part of the crtE gene).

- 5 **Figure 22:** Schematic representation of the integration sites of three transformed *Bacillus subtilis* strains: BS1012::SFCO, BS1012::SFCOCAT1 and BA1012::SFCONEO1. Amplification of the synthetic *Flavobacterium* carotenoid operon (SFCO) can only be obtained in those strains having amplifiable structures. Probe A was used to determine the copy number of the integrated SFCO. Erythromycin resistance gene (ermAM), chloramphenicol resistance gene (cat), neomycin resistance gene (neo), terminator of the cryT gene of *B. subtilis* (cryT), levan-sucrase gene (sac-B 5' and sac-B 3'), plasmid sequences of pXI12 (pXI12), promoter originating from site I of the veg promoter complex (PvegI).

**Figure 23:** Construction of plasmids pXI12-ZYIB-EINV4MUTRBS2CNEO and pXI12-ZYIB-EINV4MUTRBS2CCAT.

- 15 **Figure 24:** Complete nucleotide sequence of plasmid pZea4.

**Figure 25:** Synthetic crtW gene of *Alcaligenes* PC-1. The translated protein sequence is shown above the double stranded DNA sequence. The twelve oligonucleotides (crtW1-crtW12) used for the PCR synthesis are underlined.

- 20 **Figure 26:** Construction of plasmid pBIIKS-crtEBIYZW. The HindIII-Pm1I fragment of pALTER-Ex2-crtW, carrying the synthetic crtW gene, was cloned into the HindIII and MluI (blunt) sites. PvegI and Ptac are the promoters used for the transcription of the two operons. The ColE1 replication origin of this plasmid is compatible with the p15A origin present in the pALTER-Ex2 constructs.

- 25 **Figure 27:** Relevant inserts of all plasmids constructed in Example 7. Disrupted genes are shown by //. Restriction sites: S=SacI, X=XbaI, H=HindIII, N=NsiI, Hp=HpaI, Nd=NdeI.

- Figure 28:** Reaction products (carotenoids) obtained from  $\beta$ -carotene by the process of the present invention.

## 30 **Example 1**

### **Materials and general methods used**

- 35 **Bacterial strains and plasmids:** *Flavobacterium* sp. R1534 WT (ATCC 21588) was the DNA source for the genes cloned. Partial genomic libraries of *Flavobacterium* sp. R1534 WT DNA were constructed into the pBluescriptII+ (KS) or (SK) vector (Stratagene, La Jolla, USA) and transformed into *E. coli* XL-1 blue (Stratagene) or JM109.

- Media and growth conditions:** Transformed *E. coli* were grown in Luria broth (LB) at 37° C with 100mg Ampicillin (Amp)/ml for selection. *Flavobacterium* sp. R1534 WT was grown at 27° C in medium containing 1% glucose, 1% tryptone (Difco Laboratories), 1% yeast extract (Difco), 0.5% MgSO<sub>4</sub> 7H<sub>2</sub>O and 3% NaCl.

- 40 **Colony screening:** Screening of the *E. coli* transformants was done by PCR basically according to the method described by Zon et al. [Zon et al., *BioTechniques* 7, 696-698 (1989)] using the following primers:

- Primer #7: 5'-CCTGGATGACGTGCTGGAATATTCC-3'  
45 Primer #8: 5'-CAAGGCCCAGATCGCAGGCG-3'

- Genomic DNA:** A 50 ml overnight culture of *Flavobacterium* sp. R1534 was centrifuged at 10,000 g for 10 minutes. The pellet was washed briefly with 10 ml of lysis buffer (50 mM EDTA, 0.1M NaCl pH7.5), resuspended in 4 ml of the same buffer supplemented with 10 mg of lysozyme and incubated at 37° C for 15 minutes. After addition of 0.3 ml of N-Lauroyl sarcosine (20%) the incubation at 37° C was continued for another 15 minutes before the extraction of the DNA with phenol, phenol/chloroform and chloroform. The DNA was ethanol precipitated at room temperature for 20 minutes in the presence of 0.3 M sodium acetate (pH 5.2), followed by centrifugation at 10,000 g for 15 minutes. The pellet was rinsed with 70% ethanol, dried and resuspended in 1 ml of TE (10 mM Tris, 1mM EDTA, pH 8.0).

- 50 All genomic DNA used in the southern blot analysis and cloning experiments was dialysed against H<sub>2</sub>O for 48 hours, using collodion bags (Sartorius, Germany), ethanol precipitated in the presence of 0.3 M sodium acetate and resuspended in H<sub>2</sub>O.

**Probe labelling:** DNA probes were labeled with (a - <sup>32</sup>P) dGTP (Amersham) by random-priming according to [Sambrook et al., s.a.].

**Probes used to screen the mini-libraries:** **Probe 46F** is a 119 bp fragment obtained by PCR using primer #7 and #8 and *Flavobacterium sp.* R1534 genomic DNA as template. This probe was proposed to be a fragment of the *Flavobacterium sp.* R1534 phytoene synthase (*crtB*) gene, since it shows significant homology to the phytoene synthase genes from other species (e.g. *E. uredovora*, *E. herbicola*). **Probe A** is a BstXI - PstI fragment of 184 bp originating from the right arm of the insert of clone 85. **Probe B** is a 397 bp XhoI - NotI fragment obtained from the left end of the insert of clone 85. **Probe C** is a 536 bp BglII - PstI fragment from the right end of the insert of clone 85. **Probe D** is a 376 bp KpnI - BstYI fragment isolated from the insert of clone 59. The localization of the individual probes is shown in figure 6.

**Oligonucleotide synthesis:** The oligonucleotides used for PCR reactions or for sequencing were synthesized with an Applied Biosystems 392 DNA synthesizer.

**Southern blot analysis:** For hybridization experiments *Flavobacterium sp.* R1534 genomic DNA (3 mg) was digested with the appropriate restriction enzymes and electrophoresed on a 0.75% agarose gel. The transfer to Zeta-Probe blotting membranes (BIO-RAD), was done as described [Southern, E.M., J. Mol. Biol. **98**, 503 (1975)]. Prehybridization and hybridization was in 7%SDS, 1% BSA (fraction V; Boehringer), 0.5M Na<sub>2</sub>HPO<sub>4</sub>, pH 7.2 at 65°C. After hybridization the membranes were washed twice for 5 minutes in 2x SSC, 1% SDS at room temperature and twice for 15 minutes in 0.1% SSC, 0.1% SDS at 65° C.

**DNA sequence analysis:** The sequence was determined by the dideoxy chain termination technique [Sanger et al., Proc. Natl. Acad. Sci. USA **74**, 5463-5467 (1977)] using the Sequenase Kit (United States Biochemical). Both strands were completely sequenced and the sequence analyzed using the GCG sequence analysis software package (Version 8.0) by Genetics Computer, Inc. [Devereux et al., Nucleic Acids. Res. **12**, 387-395 (1984)].

**Analysis of carotenoids:** *E. coli* XL-1 or JM109 cells (200 - 400 ml) carrying different plasmid constructs were grown for the times indicated in the text, usually 24 to 60 hours, in LB supplemented with 100mg Ampicillin/ml, in shake flasks at 37° C and 220 rpm.

The carotenoids present in the microorganisms were extracted with an adequate volume of acetone using a rotation homogenizer (Polytron, Kinematica AG, CH-Luzern). The homogenate was filtered through the sintered glass of a suction filter into a round bottom flask. The filtrate was evaporated by means of a rotation evaporator at 50° C using a water-jet vacuum. For the zeaxanthin detection the residue was dissolved in n-hexane/acetone (86:14) before analysis with a normalphase HPLC as described in [Weber, S. in Analytical Methods for Vitamins and Carotenoids in Feed, Keller, H.E., Editor, 83-85 (1988)]. For the detection of  $\beta$ -carotene and lycopene the evaporated extract was dissolved in n-hexane/acetone (99:1) and analysed by HPLC as described in [Hengartner et al., Helv. Chim. Acta **75**, 1848-1865 (1992)].

## Example 2

### Cloning of the *Flavobacterium sp.* R1534 carotenoid biosynthetic genes.

To identify and isolate DNA fragments carrying the genes of the carotenoid biosynthesis pathway, we used the DNA fragment 46F (see methods) to probe a Southern Blot carrying chromosomal DNA of *Flavobacterium sp.* R1534 digested with different restriction enzymes Fig. 2. The 2.4 kb XhoI/PstI fragment hybridizing to the probe seemed the most appropriate one to start with. Genomic *Flavobacterium sp.* R1534 DNA was digested with XhoI/PstI and run on a 1% agarose gel. According to a comigrating DNA marker, the region of about 2.4 kb was cut out of the gel and the DNA isolated. A XhoI/PstI mini library of *Flavobacterium sp.* R1534 genomic DNA was constructed into XhoI - PstI sites of pBluescriptIIISK(+). One hundred *E. coli* XL1 transformants were subsequently screened by PCR with primer #7 and primer #8, the same primers previously used to obtain the 119 bp fragment (46F). One positive transformant, named clone 85, was found. Sequencing of the insert revealed sequences not only homologous to the phytoene synthase (*crtB*) but also to the phytoene desaturase (*crtI*) of both *Erwinia* species *herbicola* and *uredovora*. Left and right hand genomic sequences of clone 85 were obtained by the same approach using probe A and probe B. *Flavobacterium sp.* R1534 genomic DNA was double digested with ClaI and HindIII and subjected to Southern analysis with probe A and probe B. With probe A a ClaI/HindIII fragment of approx. 1.8 kb was identified (Fig. 3A), isolated and subcloned into the ClaI/HindIII sites of pBluescriptIIKS (+). Screening of the *E. coli* XL1 transformants with probe A gave 6 positive clones. The insert of one of these positives, clone 43-3, was sequenced and showed homology to the N-terminus of *crtI* genes and to the C-terminus of *crtY* genes of both *Erwinia* species mentioned above. With probe B an approx. 9.2 kb ClaI/HindIII fragment was detected (Fig. 3B), isolated and subcloned into pBluescriptIIKS (+).

A screening of the transformants gave one positive, clone 51. Sequencing of the 5' and 3' of the insert, revealed that only the region close to the HindIII site showed relevant homology to genes of the carotenoid biosynthesis of the *Erwinia* species mentioned above (e.g. *crtB* gene and *crtE* gene). The sequence around the ClaI site showed no homology to known genes of the carotenoid biosynthesis pathway. Based on this information and to facilitate further sequencing and construction work, the 4.2 kb BamHI/HindIII fragment of clone 51 was subcloned into the respective sites of pBluescriptIIKS(+) resulting in clone 2. Sequencing of the insert of this clone confirmed the presence of genes homol-

ogous to *Erwinia* sp. crtB and crtE genes. These genes were located within 1.8 kb from the HindIII site. The remaining 2.4 kb of this insert had no homology to known carotenoid biosynthesis genes.

Additional genomic sequences downstream of the ClaI site were detected using probe C to hybridize to *Flavobacterium* sp. R1534 genomic DNA digested with different restriction enzymes (see figure 4).

A SalI/HindIII fragment of 2.8 kb identified by Southern analysis was isolated and subcloned into the HindIII/XhoI sites of pBluescriptIIKS (+). Screening of the *E. coli* XL1 transformants with probe A gave one positive clone named clone 59. The insert of this clone confirmed the sequence of clone 43-3 and contained in addition sequences homologous to the N-terminus of the crtY gene from other known lycopene cyclases. To obtain the putative missing crtZ gene a Sau3AI partial digestion library of *Flavobacterium* sp. R1534 was constructed into the BamHI site of pBluescriptIIKS(+). Screening of this library with probe D gave several positive clones. One transformant designated, clone 6a, had an insert of 4.9 kb. Sequencing of the insert revealed besides the already known sequences coding for crtB, crtI and crtY also the missing crtZ gene. Clone 7g was isolated from a mini library carrying BclI/SphI fragments of R1534 (Fig. 5) and screened with probe D. The insert size of clone 7g is approx. 3 kb.

The six independent inserts of the clones described above covering approx. 14 kb of the *Flavobacterium* sp. R1534 genome are compiled in Figure 6.

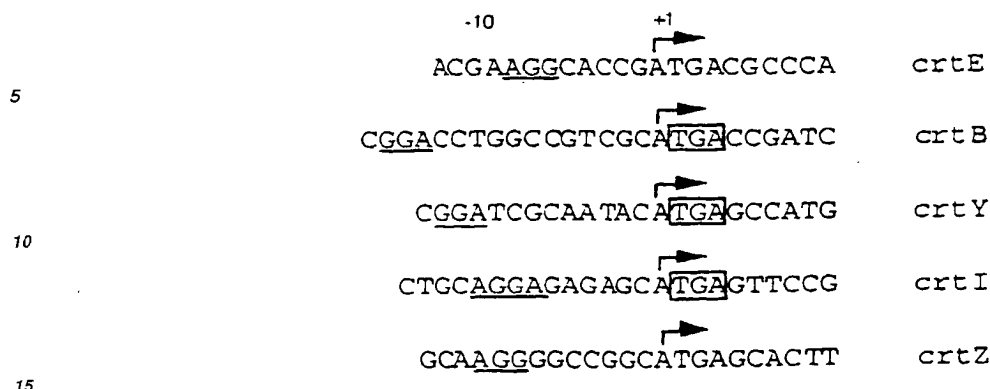
The determined sequence spanning from the BamHI site (position 1) to base pair 8625 is shown figure 7.

#### Putative protein coding regions of the cloned R1534 sequence.

Computer analysis using the CodonPreference program of the GCG package, which recognizes protein coding regions by virtue of the similarity of their codon usage to a given codon frequency table, revealed eight open reading frames (ORFs) encoding putative proteins: a partial ORF from 1 to 1165 (ORF-5) coding for a polypeptide larger than 41382 Da, an ORF coding for a polypeptide with a molecular weight of 40081 Da from 1180 to 2352 (ORF-1); an ORF coding for a polypeptide with a molecular weight of 31331 Da from 2521 to 3405 (crtE); an ORF coding for a polypeptide with a molecular weight of 32615 Da from 4316 to 3408 (crtB); an ORF coding for a polypeptide with a molecular weight of 54411 Da from 5797 to 4316 (crtI); an ORF coding for a polypeptide with a molecular weight of 42368 Da from 6942 to 5797 (crtY), an ORF coding for a polypeptide with a molecular weight of 19282 Da from 7448 to 6942 (crtZ); and an ORF coding for a polypeptide with a molecular weight of 19368 Da from 8315 to 7770 (ORF-16); ORF-1 and crtE have the opposite transcriptional orientation from the others (Fig. 6). The translation start sites of the ORFs crtI, crtY and crtZ could clearly be determined based on the appropriately located sequences homologous to the Shine/Dalgarno (S/D) [Shine and Dalgarno, Proc. Natl. Acad. Sci. USA 71, 1342-1346 (1974)] consensus sequence AGG-6-9N-ATG (Fig. 10) and the homology to the N-terminal sequences of the respective enzymes of *E. herbicola* and *E. uredovora*. The translation of the ORF crtB could potentially start from three closely spaced codons ATG (4316), ATG (4241) and ATG (4211). The first one, although not having the best S/D sequence of the three, gives a translation product with the highest homology to the N-terminus of the *E. herbicola* and *E. uredovora* crtB protein, and is therefore the most likely translation start site. The translation of ORF crtE could potentially start from five different start codons found within 150 bp : ATG (2389), ATG (2446), ATG (2473), ATG (2497) and ATG (2521). We believe that based on the following observations, the ATG (2521) is the most likely transcription start site of crtE: this ATG start codon is preceded by the best consensus S/D sequence of all five putative start sites mentioned; and the putative N-terminal amino acid sequence of the protein encoded has the highest homology to the N-terminus of the crtE enzymes of *E. herbicola* and *E. uredovora*;

#### Characteristics of the crt translational initiation sites and gene products.

The translational start sites of the five carotenoid biosynthesis genes are shown below and the possible ribosome binding sites are underlined. The genes crtZ, crtY, crtI and crtB are grouped so tightly that the TGA stop codon of the anterior gene overlaps the ATG of the following gene. Only three of the five genes (crtI, crtY and crtZ) fit with the consensus for optimal S/D sequences. The boxed TGA sequence shows the stop codon of the anterior gene.



## 20 Amino acid sequences of individual crt genes of *Flavobacterium sp.* R1534.

All five ORFs of *Flavobacterium sp.* R1534 having homology to known carotenoid biosynthesis genes of other species are clustered in approx. 5.2 kb of the sequence (Fig. 7).

### 25 GGDP synthase (crtE)

The amino acid (aa) sequence of the geranylgeranyl pyrophosphate synthase (crtE gene product) consists of 295 aa and is shown in figure 8. This enzyme condenses farnesyl pyrophosphate and isopentenyl pyrophosphate in a 1' - 4.

### 30 Phytoene synthase (crtB)

This enzyme catalyzes two enzymatic steps. First it condenses in a head to head reaction two geranylgeranyl pyrophosphates (C20) to the C40 carotenoid prephytoene. Second it rearranges the cyclopropylring of prephytoene to phytoene. The 303 aa encoded by the crtB gene of *Flavobacterium sp.* R1534 is shown in figure 9.

### 35 Phytoene desaturase (crtI)

The phytoene desaturase of *Flavobacterium sp.* R1534 consisting of 494 aa, shown in figure 10, performs like the crtI enzyme of *E. herbicola* and *E. uredovora*, four desaturation steps, converting the non-coloured carotenoid phytoene to the red coloured lycopene. **Lycopene cyclase (crtY)**

The crtY gene product of *Flavobacterium sp.* R1534 is sufficient to introduce the b-ionone rings at both sides of lycopene to obtain  $\beta$ -carotene. The lycopene cyclase of *Flavobacterium sp.* R1534 consists of 382 aa (Fig. 11).  **$\beta$ -carotene hydroxylase (crtZ)**

The gene product of crtZ consisting of 169 aa (Fig. 12) and hydroxylates  $\beta$ -carotene to the xanthophyll zeaxanthin.

## 45 Putative enzymatic functions of the ORF's (orf-1, orf-5 and orf-16)

The orf-1 has at the aa level over 40% identity to acetoacetyl-CoA thiolases of different organisms (e.g. *Candida tropicalis*, human, rat). This gene is therefore most likely a putative acetoacetyl-CoA thiolase (acetyl-CoA acetyltransferase), which condenses two molecules of acetyl-CoA to Acetoacetyl-CoA. Condensation of acetoacetyl-CoA with a third acetyl-CoA by the HMG-CoA synthase forms  $\beta$ -hydroxy- $\beta$ -methylglutaryl-CoA (HMG-CoA). This compound is part of the mevalonate pathway which produces besides sterols also numerous kinds of isoprenoids with diverse cellular functions. In bacteria and plants, the isoprenoid pathway is also able to synthesize some unique products like carotenoids, growth regulators (e.g. in plants gibberellins and abscisic acid) and secondary metabolites like phytoalexins [Riou et al., Gene 148, 293-297 (1994)].

The orf-5 has a low homology of approx. 30%, to the amino acid sequence of polyketide synthases from different streptomycetes (e.g. *S. violaceoruber*, *S. cinnamonensis*). These antibiotic synthesizing enzymes (polyketide synthases), have been classified into two groups. Type-I polyketide synthases are large multifunctional proteins, whereas type-II

polyketide synthases are multiprotein complexes composed of several individual proteins involved in the subreactions of the polyketide synthesis [Bibb, et al. *Gene* **142**, 31-39 (1994)].

The putative protein encoded by the orf-16 has at the aa level an identity of 42% when compared to the soluble hydrogenase subunit of *Anabaena cylindrica*.

### Functional assignment of the ORF 's (crtE, crtB, crtI, crtY and crtZ) to enzymatic activities of the carotenoid biosynthesis pathway.

The biochemical assignment of the gene products of the different ORF's were revealed by analyzing carotenoid accumulation in *E. coli* host strains that were transformed with deleted variants of the *Flavobacterium sp.* gene cluster and thus expressed not all of the crt genes (Fig. 13).

Three different plasmid were constructed: pLyco, p59-2 and pZea4. Plasmid p59-2 was obtained by subcloning the HindIII/BamHI fragment of clone 2 into the HindIII/BamHI sites of clone 59. p59-2 carries the ORF's of the crtE, crtB, crtI and crtY gene and should lead to the production of  $\beta$ -carotene. pLyco was obtained by deleting the KpnI/KpnI fragment, coding for approx. one half (N-terminus) of the crtY gene, from the p59-2 plasmid. *E. coli* cells transformed with pLyco, and therefore having a truncated non-functional crtY gene, should produce lycopene, the precursor of  $\beta$ -carotene. pZea4 was constructed by ligation of the AscI-SpeI fragment of p59-2, containing the crtE, crtB, crtI and most of the crtY gene with the AscI/XbaI fragment of clone 6a, containing the sequences to complete the crtY gene and the crtZ gene. pZea4 [for complete sequence see Fig. 24; nucleotides 1 to 683 result from pBluescriptIIKS(+), nucleotides 684 to 8961 from *Flavobacterium* R1534 WT genome, nucleotides 8962 to 11233 from pBluescriptIIKS(+)] has therefore all five ORF's of the zeaxanthin biosynthesis pathway. Plasmid pZea4 has been deposited on May 25, 1995 at the DSM-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Germany) under accession No. DSM 10012. *E. coli* cells transformed with this latter plasmid should therefore produce zeaxanthin. For the detection of the carotenoid produced, transformants were grown for 48 hours in shake flasks and then subjected to carotenoid analysis as described in the methods section. Figure 13 summarizes the different inserts of the plasmids described above, and the main carotenoid detected in the cells.

As expected the pLyco carrying *E. coli* cells produced lycopene, those carrying p59-2 produced  $\beta$ -carotene (all-E,9-Z,13-Z) and the cells having the pZea4 construct produced zeaxanthin. This confirms that all the necessary genes of *Flavobacterium sp.* R1534 for the synthesis of zeaxanthin or their precursors (phytoene, lycopene and  $\beta$ -carotene) were cloned.

### Example 3

#### Materials and methods used for expression of carotenoid synthesizing enzymes

**Bacterial strains and plasmids:** The vectors pBluescriptIIKS (+) or (-) (Stratagene, La Jolla, USA) and pUC18 [Vieira and Messing, *Gene* **19**, 259-268 (1982); Norrander et al., *Gene* **26**, 101-106 (1983)] were used for cloning in different *E. coli* strains, like XL-1 blue (Stratagene), TG1 or JM109. In all *B. subtilis* transformations, strain 1012 was used. Plasmids pHP13 [Haima et al., *Mol. Gen. Genet.* **209**, 335-342 (1987)] and p602/22 [LeGrice, S.F.J. in *Gene Expression Technology*, Goeddel, D.V., Editor, 201-214 (1990)] are Gram (+)/(-) shuttle vectors able to replicate in *B. subtilis* and *E. coli* cells. Plasmid p205 contains the vegI promoter cloned into the SmaI site of pUC18. Plasmid pXI12 is an integration vector for the constitutive expression of genes in *B. subtilis* [Haiker et al., in 7th Int. Symposium on the Genetics of Industrial Microorganisms, June 26-July 1, 1994. Mongreal, Quebec, Canada (1994)]. Plasmid pBEST501 [Itaya et al., *Nucleic Acids Res.* **17** (11), 4410 (1989)] contains the neomycin resistance gene cassette originating from the plasmid pUB110 (GenBank entry: M19465) of *S. aureus* [McKenzie et al., *Plasmid* **15**, 93-103 (1986); McKenzie et al., *Plasmid* **17**, 83-84 (1987)]. This neomycin gene has been shown to work as a selection marker when present in a single copy in the genome of *B. subtilis*. Plasmid pC194 (ATCC 37034)(GenBank entry: L08860) originates from *S. aureus* [Horinouchi and Weisblum, *J. Bacteriol.* **150**, 815-825 (1982)] and contains the chloramphenicol acetyltransferase gene.

**Media and growth conditions:** *E. coli* were grown in Luria broth (LB) at 37° C with 100mg Ampicillin (Amp)/ml for selection. *B. subtilis* cells were grown in VY-medium supplemented with either erythromycin (1 mg/ml), neomycin (5-180 mg/ml) or chloramphenicol (10-80 mg/ml).

**Transformation:** *E. coli* transformations were done by electroporation using the Gene-pulser device of BIO-RAD (Hercules, CA, USA) with the following parameters (200 W, 250 mFD, 2.5V). *B. subtilis* transformations were done basically according to the standard procedure method 2.8 described by [Cutting and Vander Horn in *Molecular Biological Methods for Bacillus*, Harwood, C.R. and Cutting, S.M., Editor, John Wiley & Sons: Chichester, England. 61-74 (1990)].

**Colony screening:** Bacterial colony screening was done as described by [Zon et al., s.a.].

**Oligonucleotide synthesis:** The oligonucleotides used for PCR reactions or for sequencing were synthesized with

an Applied Biosystems 392 DNA synthesizer.

**PCR reactions:** The PCR reactions were performed using either the **UITma DNA polymerase** (Perkin Elmer Cetus) or the **Pfu Vent polymerase** (New England Biolabs) according to the manufacturers instructions. A typical 50 ml PCR reaction contained: 100ng of template DNA, 10 pM of each of the primers, all four dNTP's (final conc. 300 mM), MgCl<sub>2</sub> (when UITma polymerase was used; final conc. 2 mM), 1x UITma reaction buffer or 1x Pfu buffer (supplied by the manufacturer). All components of the reaction with the exception of the DNA polymerase were incubated at 95°C for 2 min. followed by the cycles indicated in the respective section (see below). In all reactions a hot start was made, by adding the polymerase in the first round of the cycle during the 72°C elongation step. At the end of the PCR reaction an aliquot was analysed on 1% agarose gel, before extracting once with phenol/chloroform. The amplified fragment in the aqueous phase was precipitated with 1/10 of a 3M NaAcetate solution and two volumes of Ethanol. After centrifugation for 5 min. at 12000 rpm, the pellet was resuspended in an adequate volume of H<sub>2</sub>O, typically 40 ml, before digestion with the indicated restriction enzymes was performed. After the digestion the mixture was separated on a 1% low melting point agarose. The PCR product of the expected size were excised from the agarose and purified using the glass beads method (GENECLEAN KIT, Bio 101, Vista CA, USA) when the fragments were above 400 bp or directly spun out of the gel when the fragments were shorter than 400 bp as described by [Heery et al., TIBS 6 (6), 173 (1990)].

#### Oligos used for gene amplification and site directed mutagenesis:

All PCR reactions performed to allow the construction of the different plasmids are described below. All the primers used are summarized in figure 14.

**Primers #100 and #101** were used in a PCR reaction to amplify the complete crtE gene having a SpeI restriction site and an artificial ribosomal binding site (RBS) upstream of the transcription start site of this gene. At the 3' end of the amplified fragment, two unique restriction sites were introduced, an AvrII and a SmaI site, to facilitate the further cloning steps. The PCR reaction was done with UITma polymerase using the following conditions for the amplification: 5 cycles with the profile: 95°C, 1 min./ 60°C, 45 sec./ 72°C, 1 min. and 20 cycles with the profile: 95°C, 1 min./ 72°C, 1 min.. Plasmid pBIIKS(+)-clone2 served as template DNA. The final PCR product was digested with SpeI and SmaI and isolated using the GENECLEAN KIT. The size of the fragment was approx. 910 bp.

**Primers #104 and #105** were used in a PCR reaction to amplify the crtZ gene from the translation start till the Sall restriction site, located in the coding sequence of this gene. At the 5' end of the crtZ gene an EcoRI, a synthetic RBS and a NdeI site was introduced. The PCR conditions were as described above. Plasmid pBIIKS(+)-clone 6a served as template DNA and the final PCR product was digested with EcoRI and Sall. Isolation of the fragment of approx. 480 bp was done with the GENECLEAN KIT.

**Primers MUT1 and MUT5** were used to amplify the complete crtY gene. At the 5' end, the last 23 nucleotides of the crtZ gene including the Sall site are present, followed by an artificial RBS preceding the translation start site of the crtY gene. The artificial RBS created includes a PmlI restriction site. The 3' end of the amplified fragment contains 22 nucleotides of the crtI gene, preceded by an newly created artificial RBS which contains a MunI restriction site. The conditions used for the PCR reaction were as described above using the following cycling profile: 5 rounds of 95°C, 45 sec./ 60°C, 45 sec./ 72°C, 75 sec. followed by 22 cycles with the profile: 95°C, 45 sec./ 66°C, 45 sec./ 72°C, 75 sec.. Plasmid pXI12-ZYIB-EINV4 served as template for the Pfu Vent polymerase. The PCR product of 1225 bp was made blunt and cloned into the SmaI site of pUC18, using the Sure-Clone Kit (Pharmacia) according to the manufacturer.

**Primers MUT2 and MUT6** were used to amplify the complete crtI gene. At the 5' the last 23 nucleotides of the crtY gene are present, followed by an artificial RBS which precedes the translation start site of the crtI gene. The new RBS created, includes a MunI restriction site. The 3' end of the amplified fragment contains the artificial RBS upstream of the crtB gene including a BamHI restriction site. The conditions used for the PCR reaction were basically as described above including the following cycling profile: 5 rounds of 95°C, 30 sec./ 60°C, 30 sec./ 72°C, 75 sec., followed by 25 cycles with the profile: 95°C, 30 sec./ 66°C, 30 sec./ 72°C, 75 sec.. Plasmid pXI12-ZYIB-EINV4 served as template for the Pfu Vent polymerase. For the further cloning steps the PCR product of 1541 bp was digested with MunI and BamHI.

**Primers MUT3 and CAR17** were used to amplify the N-terminus of the crtB gene. At the 5' the last 28 nucleotides of the crtI gene are present followed by an artificial RBS, preceding the translation start site of the crtB gene. This new created RBS, includes a BamHI restriction site. The amplified fragment, named PCR-F contains also the HindIII restriction site located at the N-terminus of the crtB gene. The conditions used for the PCR reaction were as described elsewhere in the text, including the following cycling profile: 5 rounds of 95°C, 30 sec./ 58°C, 30 sec./ 72°C, 20 sec. followed by 25 cycles with the profile: 95°C, 30 sec./ 60°C, 30 sec./ 72°C, 20 sec.. Plasmid pXI12-ZYIB-EINV4 served as template for the Pfu Vent polymerase. The PCR product of approx. 160 bp was digested with BamHI and HindIII.

#### Oligos used to amplify the chloramphenicol resistance gene (cat):

**Primers CAT3 and CAT4** were used to amplify the chloramphenicol resistance gene of pC194 (ATCC 37034) [Hori-

nouchi and Weisblum, s.a.] a R-plasmid found in *S. aureus*. The conditions used for the PCR reaction were as described previously including the following cycling profile: 5 rounds of 95°C, 60 sec./ 50°C, 60 sec./ 72°C, 2 min. followed by 20 cycles with the profile: 95°C, 60 sec./ 60°C, 60 sec./ 72°C, 2 min.. Plasmid pC198 served as template for the Pfu Vent polymerase. The PCR product of approx. 1050 bp was digested with EcoRI and AatII.

**Oligos used to generate linkers:** Linkers were obtained by adding 90 ng of each of the two corresponding primers into an Eppendorf tube. The mixture was dried in a speed vac and the pellet resuspended in 1x Ligation buffer (Boehringer, Mannheim, Germany). The solution was incubated at 50°C for 3 min. before cooling down to RT, to allow the primers to hybridize properly. The linker were now ready to be ligated into the appropriate sites. All the oligos used to generate linkers are shown in figure 15.

Primers **CS1** and **CS2** were used to form a linker containing the following restrictions sites HindIII, AflII, ScaI, XbaI, Pml and EcoRI.

Primers **MUT7** and **MUT8** were used to form a linker containing the restriction sites Sall, AvrII, PmlI, MluI, MunI, BamHI, SphI and HindIII.

Primers **MUT9** and **MUT10** were used to introduce an artificial RBS upstream of crtY.

Primers **MUT11** and **MUT12** were used to introduce an artificial RBS upstream of crtE.

**Isolation of RNA:** Total RNA was prepared from log phase growing *B. subtilis* according to the method described by [Maes and Messens, Nucleic Acids Res. 20 (16), 4374 (1992)].

**Northern Blot analysis:** For hybridization experiments up to 30 mg of *B. subtilis* RNA was electrophoresed on a 1% agarose gel made up in 1x MOPS and 0.66 M formaldehyde. Transfer to Zeta-Probe blotting membranes (BIO-RAD), UV cross-linking, pre-hybridization and hybridization was done as described elsewhere in [Farrell, J.R.E., RNA Methodologies. A laboratory Guide for isolation and characterization. San Diego, USA: Academic Press (1993)]. The washing conditions used were: 2 x 20 min. in 2xSSPE/0.1% SDS followed by 1 x 20 min. in 0.1% SSPE/0.1% SDS at 65°C. Northern blots were then analyzed either by a Phosphorimager (Molecular Dynamics) or by autoradiography on X-ray films from Kodak.

**Isolation of genomic DNA:** *B. subtilis* genomic DNA was isolated from 25 ml overnight cultures according to the standard procedure method 2.6 described by [13].

**Southern blot analysis:** For hybridization experiments *B. subtilis* genomic DNA (3 mg) was digested with the appropriate restriction enzymes and electrophoresed on a 0.75% agarose gel. The transfer to Zeta-Probe blotting membranes (BIO-RAD), was done as described [Southern, E.M., s.a.]. Prehybridization and hybridization was in 7%SDS, 1% BSA (fraction V; Boehringer), 0.5M Na<sub>2</sub>HPO<sub>4</sub>, pH 7.2 at 65°C. After hybridization the membranes were washed twice for 5 min. in 2x SSC, 1% SDS at room temperature and twice for 15 min. in 0.1% SSC, 0.1% SDS at 65°C. Southern blots were then analyzed either by a Phosphorimager (Molecular Dynamics) or by autoradiography on X-ray films from Kodak.

**DNA sequence analysis:** The sequence was determined by the dideoxy chain termination technique [Sanger et al., s.a.] using the Sequenase Kit Version 1.0 (United States Biochemical). Sequence analysis were done using the GCG sequence analysis software package (Version 8.0) by Genetics Computer, Inc. [Devereux et al., s.a.].

**Gene amplification in *B. subtilis*:** To amplify the copy number of the SFCO in *B. subtilis* transformants, a single colony was inoculated in 15 ml VY-medium supplemented with 1.5 % glucose and 0.02 mg chloramphenicol or neomycin/ml, dependend on the antibiotic resistance gene present in the amplifiable structure (see results and discussion). The next day 750 ml of this culture were used to inoculate 13 ml VY-medium containing 1.5% glucose supplemented with (60, 80, 120 and 150 mg/ml) for the cat resistant mutants, or 160 mg/ml and 180 mg/ml for the neomycin resistant mutants). The cultures were grown overnight and the next day 50 ml of different dilutions (1: 20, 1:400; 1: 8000, 1: 160'000) were plated on VY agar plates with the appropriate antibiotic concentration. Large single colonies were then further analyzed to determine the number of copies and the amount of carotenoids produced.

**Analysis of carotenoids:** *E. coli* or *B. subtilis* transformants (200 - 400 ml) were grown for the times indicated in the text, usually 24 to 72 hours, in LB-medium or VY-medium, respectively, supplemented with antibiotics, in shake flasks at 37° C and 220 rpm.

The carotenoids produced by the microorganisms were extracted with an adequate volume of acetone using a rotation homogenizer (Polytron, Kinematica AG, CH-Luzern). The homogenate was filtered through the sintered glass of a suction filter into a round bottom flask. The filtrate was evaporated by means of a rotation evaporator at 50° C using a water-jet vacuum. For the zeaxanthin detection the residue was dissolved in n-hexane/acetone (86:14) before analysis with a normalphase HPLC as described in [Weber, S., s.a.]. For the detection of  $\beta$ -carotene and lycopene the evaporated extract was dissolved in n-hexane/acetone (99:1) and analysed by HPLC as described in Hengartner et al., s.a.].

**Example 4****Carotenoid production in *E. coli***

The biochemical assignment of the gene products of the different open reading frames (ORF's) of the carotenoid biosynthesis cluster of *Flavobacterium sp.* were revealed by analyzing the carotenoid accumulation in *E. coli* host strains, transformed with plasmids carrying deletions of the *Flavobacterium sp.* gene cluster, and thus lacking some of the crt gene products. Similar functional assays in *E. coli* have been described by other authors [Misawa et al., s.a.; Perry et al., J. Bacteriol., 168, 607-612 (1986); Hundle, et al., Molecular and General Genetics 254 (4), 406-416 (1994)].

Three different plasmid pLyco, pBIIKS(+)-clone59-2 and pZea4 were constructed from the three genomic isolates pBIIKS(+)-clone2, pBIIKS(+)-clone59 and pBIIKS(+)-clone6a (see figure 16).

Plasmid pBIIKS(+)-clone59-2 was obtained by subcloning the HindIII/BamHI fragment of pBIIKS(+)-clone 2 into the HindIII/BamHI sites of pBIIKS(+)-clone59. The resulting plasmid pBIIKS(+)-clone59-2 carries the complete ORF's of the crtE, crtB, crtI and crtY gene and should lead to the production of  $\beta$ -carotene. pLyco was obtained by deleting the KpnI/KpnI fragment, coding for approx. one half (N-terminus) of the crtY gene, from the plasmid pBIIKS(+)-clone59-2. *E. coli* cells transformed with pLyco, and therefore having a truncated non-functional crtY gene, should produce lycopene, the precursor of  $\beta$ -carotene. pZea4 was constructed by ligation of the AscI-SpeI fragment of pBIIKS(+)-clone59-2, containing the crtE, crtB, crtI and most of the crtY gene with the AscI/XbaI fragment of clone 6a, containing the crtZ gene and sequences to complete the truncated crtY gene mentioned above. pZea4 has therefore all five ORF's of the zeaxanthin biosynthesis pathway. *E. coli* cells transformed with this latter plasmid should therefore produce zeaxanthin. For the detection of the carotenoid produced, transformants were grown for 43 hours in shake flasks and then subjected to carotenoid analysis as described in the methods section. Figure 16 summarizes the construction of the plasmids described above.

As expected the pLyco carrying *E. coli* cells produced lycopene, those carrying pBIIKS(+)-clone59-2 produced  $\beta$ -carotene (all-E,9-Z,13-Z) and the cells having the pZea4 construct produced zeaxanthin. This confirms that we have cloned all the necessary genes of *Flavobacterium sp.* R1534 for the synthesis of zeaxanthin or their precursors (phytoene, lycopene and  $\beta$ -carotene). The production levels obtained are shown in table 1.

plasmid	host	zeaxanthin	$\beta$ -carotene	lycopene
pLyco	<i>E. coli</i> JM109	ND	ND	0.05%
pBIIKS(+)-clone59-2	"	ND	0.03%	ND
pZea4	"	0.033%	0.0009%	ND

Table 1: Carotenoid content of *E. coli* transformants, carrying the plasmids pLyco, pBIIKS(+)-clone59-2 and pZea4, after 43 hours of culture in shake flasks. The values indicated show the carotenoid content in % of the total dry cell mass (200 ml). ND = not detectable.

**Examples 5****Carotenoid production in *B. subtilis***

In a first approach to produce carotenoids in *B. subtilis*, we cloned the carotenoid biosynthesis genes of *Flavobacterium* into the Gram (+)/(-) shuttle vectors p602/22, a derivative of p602/20 [LeGrice, S.F.J., s.a.]. The assembling of the final construct p602-CARVEG-E, begins with a triple ligation of fragments PvuII-AvrII of pZea4(del654-

3028) and the AvrII-EcoRI fragment from plasmid pBIKS(+)-clone6a, into the EcoRI and ScaI sites of the vector p602/22. The plasmid pZea4(del654-3028) had been obtained by digesting pZea4 with SacI and EspI. The protruding and recessed ends were made blunt with Klenow enzyme and religated. Construct pZea4(del654-3028) lacks most of the sequence upstream of crtE gene, which are not needed for the carotenoid biosynthesis. The plasmid p602-CAR has approx. 6.7 kb of genomic *Flavobacterium R1534* DNA containing besides all five carotenoid genes (approx. 4.9 kb), additional genomic DNA of 1.2 kb, located upstream of the crtZ translation start site and further 200 bp, located upstream of crtE transcription start. The crtZ, crtY, crtI and crtB genes were cloned downstream of the P<sub>N25/0</sub> promoter, a regulatable *E. coli* bacteriophage T5 promoter derivative, fused to a lac operator element, which is functional in *B. subtilis* [LeGrice, S.F.J., s.a.]. It is obvious that in the p602CAR construct, the distance of over 1200 bp between the P<sub>N25/0</sub> promoter and the transcription start site of crtZ is not optimal and will be improved at a later stage. An outline of the p602CAR construction is shown in figure 17. To ensure transcription of the crtE gene in *B. subtilis*, the vegI promoter [Moran et al., Mol. Gen. Genet. 186, 339-346 (1982); LeGrice et al., Mol. Gen. Genet. 204, 229-236 (1986)] was introduced upstream of this gene, resulting in the plasmid construct p602-CARVEG-E. The vegI promoter, which originates from site of the veg promoter complex described by [LeGrice et al., s.a.] has been shown to be functional in *E. coli* [Moran et al., s.a.]. To obtain this new construct, the plasmid p602CAR was digested with Sall and HindIII, and the fragment containing the complete crtE gene and most of the crtB coding sequence, was subcloned into the XhoI and HindIII sites of plasmid p205. The resulting plasmid p205CAR contains the crtE gene just downstream of the PvegI promoter. To reconstitute the carotenoid gene cluster of *Flavobacterium sp.* the following three pieces were isolated: PmeI/HindIII fragment of p205CAR, the HincII/XbaI fragment and the EcoRI/HindIII fragment of p602CAR and ligated into the EcoRI and XbaI sites of pBluescriptIIKS(+), resulting in the construct pBIKS(+)-CARVEG-E. Isolation of the EcoRI-XbaI fragment of this latter plasmid and ligation into the EcoRI and XbaI sites of p602/22 gives a plasmid similar to p602CAR but having the crtE gene driven by the PvegI promoter. All the construction steps to get the plasmid p602CARVEG-E are outlined in figure 18. *E. coli* TG1 cells transformed with this plasmid synthesized zeaxanthin. In contrast *B. subtilis* strain 1012 transformed with the same constructs did not produce any carotenoids. Analysis of several zeaxanthin negative *B. subtilis* transformants always revealed, that the transformed plasmids had undergone severe deletions. This instability could be due to the large size of the constructs.

In order to obtain a stable construct in *B. subtilis*, the carotenoid genes were cloned into the Gram (+)/(-) shuttle vector pHP13 constructed by [Haima et al., s.a.]. The stability problems were thought to be omitted by 1) reducing the size of the cloned insert which carries the carotenoid genes and 2) reversing the orientation of the crtE gene and thus only requiring one promoter for the expression of all five genes, instead of two, like in the previous constructs. Furthermore, the ability of cells transformed by such a plasmid carrying the synthetic *Flavobacterium* carotenoid operon (SFCO), to produce carotenoids, would answer the question if a modular approach is feasible. Figure 19 summarizes all the construction steps and intermediate plasmids made to get the final construct pHP13-2PNZYIB-EINV. Briefly: To facilitate the following constructions, a vector pHP13-2 was made, by introducing a synthetic linker obtained with primer CS1 and CS2, between the HindIII and EcoRI sites of the shuttle vector pHP13. The intermediate construct pHP13-2CARVEG-E was constructed by subcloning the AflII-XbaI fragment of p602CARVEG-E into the AflII and XbaI sites of pHP13-2. The next step consisted in the inversion of crtE gene, by removing XbaI and AvrII fragment containing the original crtE gene and replacing it with the XbaI-AvrII fragment of plasmid pBIKS(+)-PCRRBSrtE. The resulting plasmid was named pHP13-2CARZYIB-EINV and represented the first construction with a functional SFCO. The intermediate construct pBIKS(+)-PCRRBSrtE mentioned above, was obtained by digesting the PCR product generated with primers #100 and #101 with SpeI and SmaI and ligating into the SpeI and SmaI sites of pBluescriptIIKS(+). In order to get the crtZ transcription start close to the promoter P<sub>N25/0</sub> a triple ligation was done with the BamHI-Sall fragment of pHP13-2CARZYIB-EINV (contains four of the five carotenoid genes), the BamHI-EcoRI fragment of the same plasmid containing the P<sub>N25/0</sub> promoter and the EcoRI-Sall fragment of pBIKS(+)-PCRRBSrtZ, having most of the crtZ gene preceded by a synthetic RBS. The aforementioned plasmid pBIKS(+)-PCRRBSrtZ was obtained by digesting the PCR product amplified with primers #104 and #105 with EcoRI and Sall and ligating into the EcoRI and Sall sites of pBluescriptIIKS(+). In the resulting vector pHP13-2PN25ZYIB-EINV, the SFCO is driven by the bacteriophage T5 promoter P<sub>N25/0</sub>, which should be constitutively expressed, due to the absence of a functional lac repressor in the construct [Peschke and Beuk, J. Mol. Biol. 186, 547-555 (1985)]. *E. coli* TG1 cells transformed with this construct produced zeaxanthin. Nevertheless, when this plasmid was transformed into *B. subtilis*, no carotenoid production could be detected. Analysis of the plasmids of these transformants showed severe deletions, pointing towards instability problems, similar to the observations made with the aforementioned plasmids.

## Examples 6

### Chromosome Integration Constructs

Due to the instability observed with the previous constructs we decided to integrate the carotenoid biosynthesis

genes of *Flavobacterium sp.* into the genome of *B. subtilis* using the integration/expression vector pX112. This vector allows the constitutive expression of whole operons after integration into the levan-sucrase gene (sacB) of the *B. subtilis* genome. The constitutive expression is driven by the veg1 promoter and results in medium level expression. The plasmid pX112-ZYIB-EINV4 containing the synthetic *Flavobacterium* carotenoid operon (SFCO) was constructed as follows: the NdeI-HincII fragment of pBIISK(+)-PCRRBSctZ was cloned into the NdeI and SmaI sites of pX112 and the resulting plasmid was named pX112-PCRctZ. In the next step, the BstEII-PmeI fragment of pHP13-2PN25ZYIB-EINV was ligated to the BstEII-PmeI fragment of pX112-PCRctZ (see figure 20). *B. subtilis* transformed with the resulting construct pX112-ZYIB-EINV4 can integrate the CAR genes either via a Campbell type reaction or via a reciprocal recombination. One transformant, BS1012::ZYIB-EINV4, having a reciprocal recombination of the carotenoid biosynthesis genes into the levan-sucrase gene was further analyzed (figure 21). Although this strain did not synthesize carotenoids, RNA analysis by Northern blots showed the presence of specific polycistronic mRNA's of 5.4 kb and 4.2 kb when hybridized to probe A (see figure 21, panel B). Whereas the larger mRNA has the expected message size, the origin of the shorter mRNA was unclear. Hybridization of the same Northern blot to probe B only detected the large mRNA fragment, pointing towards a premature termination of the transcription at the end of the crtB gene. The presence of a termination signal at this location would make sense, since in the original operon organisation in the *Flavobacterium sp. R1534* genome, the crtE and the crtB genes are facing each other. With this constellation a transcription termination signal at the 5' end of crtB would make sense, in order to avoid the synthesis of anti-sense RNA which could interfere with the mRNA transcript of the crtE gene. Since this region has been changed considerably with respect to the wild type situation, the sequences constituting this terminator may also have been altered resulting in a "leaky" terminator. Western blot analysis using antisera against the different crt enzymes of the carotenoid pathway, pointed towards the possibility that the ribosomal binding sites might be responsible for the lack of carotenoid synthesis. Out of the five genes introduced only the product of crtZ, the  $\beta$ -carotene hydroxylase was detectable. This is the only gene preceded by a RBS site, originating from the pX112 vector, known to be functional in *B. subtilis*. Base pairing interactions between a mRNA's Shine-Dalgarno sequence [Shine and Delagarno, s. a.] and the 16S rRNA, which permits the ribosome to select the proper initiation site, have been proposed by [McLaughlin et al., J. Biol. Chem. 256, 11283-11291 (1981)] to be much more stable in Gram-positive organisms (*B. subtilis*) than in Gram-negative organisms (*E. coli*). In order to obtain highly stable complexes we exchanged the RBS sites of the Gram-negative *Flavobacterium sp.*, preceding each of the genes crtY, crtI, crtB and crtE, with synthetic RBS's which were designed complementary to the 3' end of the *B. subtilis* 16S rRNA (see table 2). This exchange should allow an effective translation initiation of the different carotenoid genes in *B. subtilis*. The strategy chosen to construct this pX112-ZYIB-EINV4MUTRBS2C, containing all four altered sites is summarized in figure 20. In order to facilitate the further cloning steps in pBluescriptIIKS(+), additional restriction sites were introduced using the linker obtained with primer MUT7 and MUT8, cloned between the Sall and HindIII sites of said vector. The new resulting construct pBIKS(+)-LINKER78 had the following restriction sites introduced: AvrII, PmlI, MuiI, MunI, BamHI and SphI. The general approach chosen to create the synthetic RBS's upstream of the different carotenoid genes, was done using a combination of PCR based mutagenesis, where the genes were reconstructed using defined primers carrying the modified RBS sites, or using synthetic linkers having such sequences. Reconstitution of the RBS preceding the crtI and crtB genes was done by amplifying the crtI gene with the primers MUT2 and MUT6, which include the appropriate altered RBS sites. The PCR-I fragment obtained was digested with MunI and BamHI and ligated into the MunI and BamHI sites of pBIKS(+)-LINKER78. The resulting intermediate construct was named pBIKS(+)-LINKER78PCR-I. Reconstitution of the RBS preceding the crtB gene was done using a small PCR fragment obtained with primer MUT3, carrying the altered RBS site upstream of crtB, and primer CAR17. The amplified PCR-F fragment was digested with BamHI and HindIII and sub cloned into the BamHI and HindIII sites of pBIKS(+)-LINKER78, resulting in the construct pBIKS(+)-LINKER78PCR-F. The PCR-I fragment was cut out of pBIKS(+)-LINKER78PCR-I with BamHI and SapI and ligated into the BamHI and SapI sites of pBIKS(+)-LINKER78PCR-F. The resulting plasmid pBIKS(+)-LINKER78PCR-FI has the PCR-I fragment fused to the PCR-F fragment. This construct was cut with Sall and PmlI and a synthetic linker obtained by annealing of primer MUT9 and MUT10 was introduced. This latter step was done to facilitate the upcoming replacement of the original *Flavobacterium* RBS in the above mentioned construct. The resulting plasmid was named pBIKS(+)-LINKER78PCR-FIA. Assembling of the synthetic RBS's preceding the crtY and crtI genes was done by PCR, using primers MUT1 and MUT5. The amplified fragment PCR-G was made blunt end before cloning into the SmaI site of pUC18, resulting in construct pUC18-PCR-G. The next step was the cloning of the PCR-G fragment between the PCR-A and PCR-I fragments. For this purpose the PCR-G was isolated from pUC18-PCR-G by digesting with MunI and PmlI and ligated into the MunI and PmlI sites of pBIKS(+)-LINKER78PCR-FIA. This construct contains all four fragments, PCR-F, PCR-I, PCR-G and PCR-A, assembled adjacent to each other and containing three of the four artificial RBS sites (crtY, crtI and crtB). The exchange of the *Flavobacterium* RBS's preceding the genes crtY, crtI and crtB by synthetic ones, was done by replacing the HindIII-Sall fragment of plasmid pX112-ZYIB-EINV4 with the HindIII-Sall fragment of plasmid pBIKS(+)-LINKER78PCR-FIA. The resulting plasmid pX112-ZYIB-EINV4 MUTRBS2C was subsequently transformed into *E. coli* TG1 cells and *B. subtilis* 1012. The production of zeaxanthin by these cells confirmed that the PCR amplified genes were functional. The *B.*

*subtilis* strain obtained was named BS1012::SFCO1. The last *Flavobacterium* RBS to be exchanged was the one preceding the crtE gene. This was done using a linker obtained using primer MUT11 and MUT12. The wild type RBS was removed from pXI12-ZYIB-EINV4MUTRBS with NdeI and SpeI and the above mentioned linker was inserted. In the construct pXI12-ZYIB-EINV4MUTRBS2C all *Flavobacterium* RBS's have been replaced by synthetic RBS's of the consensus sequence AAAGGAGG- 7-8 N -ATG (see table 2). *E. coli* TG1 cells transformed with this construct showed that also this last RBS replacement had not interfered

Table 2

<u>mRNA</u>	<u>nucleotide sequence</u>
crtZ	AAAGGAGGGUUUCAUAUGAGC
crtY	AAAGGAGGACACGUGAUGAGC
crtI	AAAGGAGGCAAUUGAGAUGAGU
crtB	AAAGGAGGAUCCAAUCAUGACC
crtE	AAAGGAGGGUUUCUUAUGACG

<i>B. subtilis</i>	16S rRNA	3'-UCUUUCCUCCACUAG
<i>E. coli</i>	16S rRNA	3'-AUUCCUCCACUAG

Table 2: Nucleotide sequences of the synthetic ribosome binding sites in the constructs pXI12-ZYIB-EINV4MUTRBS2C, pXI12-ZYIB-EINV4MUTRBS2CCAT and pXI12-ZYIB-EINV4MUTRBS2CNEO. Nucleotides of the Shine-Dalgarno sequence preceding the individual carotenoid genes which are complementary to the 3' ends of the 16S rRNA of *B. subtilis* are shown in bold. The 3' ends of the 16S rRNA of *E. coli* is also shown as comparison. The underlined AUG is the translation start site of the mentioned gene.

with the ability to produce zeaxanthin. All the regions containing the newly introduced synthetic RBS's were confirmed by sequencing. *B. subtilis* cells were transformed with plasmid pXI12-ZYIB-EINV4MUTRBS2 and one transformant having integrated the SFCO by reciprocal recombination, into the levan-sucrase gene of the chromosome, was selected. This strain was named BS1012::SFCO2. Analysis of the carotenoid production of this strain show that the amounts zeaxanthin produced is approx. 40% of the zeaxanthin produced by *E. coli* cells transformed with the plasmid used to get the *B. subtilis* transformant. Similar was the observation when comparing the BS1012::SFCO1 strain with its *E. coli* counter part (30%). Although the *E. coli* cells have 18 times more carotenoid genes, the carotenoid production

is only a factor of 2-3 times higher. More drastic was the difference observed in the carotenoid contents, between *E. coli* cells carrying the pZea4 construct in about 200 copies and the *E. coli* carrying the plasmid pXI12-ZYIB-EINV4MUTRBS2C in 18 copies. The first transformant produced 48x more zeaxanthin than the latter one. This difference seen can not only be attributed to the roughly 11 times more carotenoid biosynthesis genes present in these transformants. Contributing to this difference is probably also the suboptimal performance of the newly constructed SFCO, in which the overlapping genes of the wild type *Flavobacterium* operon were separated to introduce the synthetic RBS's. This could have resulted in a lower translation efficiency of the rebuild synthetic operon (e.g. due to elimination of putative translational coupling effects, present in the wild type operon).

In order to increase the carotenoid production, two new constructs were made, pXI12-ZYIB-EINV4MUTRBS2CNEO and pXI12-ZYIB-EINV4MUTRBS2CCAT, which after the integration of the SFCO into the levan-sucrase site of the chromosome, generate strains with an amplifiable structure as described by [Janniére et al., Gene 40, 47-55 (1985)]. Plasmid pXI12-ZYIB-EINV4MUTRBS2CNEO has been deposited on May 25, 1995 at the DSM-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Germany) under accession No. DSM 10013. Such amplifiable structures, when linked to a resistance marker (e.g. chloramphenicol, neomycin, tetracycline), can be amplified to 20-50 copies per chromosome. The amplifiable structure consist of the SFCO, the resistance gene and the pXI12 sequence, flanked by direct repeats of the sac-B 3' gene (see figure 22). New strains having elevated numbers of the SFCO could now be obtained by selecting for transformants with increased level of resistance to the antibiotic. To construct plasmid pXI12-ZYIB-EINV4MUTRBS2CNEO, the neomycin resistance gene was isolated from plasmid pBEST501 with PstI and SmaI and subcloned into the PstI and EcoO1091 sites of the pUC18 vector. The resulting construct was named pUC18-Neo. To get the final construct, the PmeI - AatII fragment of plasmid pXI12-ZYIB-EINV4MUTRBS2C was replaced with the SmaI-AatII fragment of pUC18-Neo, containing the neomycin resistance gene. Plasmid pXI12-ZYIB-EINV4MUTRBS2CCAT was obtained as follows: the chloramphenicol resistance gene of pC194 was isolated by PCR using the primer pair cat3 and cat4. The fragment was digested with EcoRI and AatII and subcloned into the EcoRI and AatII sites of pUC18. The resulting plasmid was named pUC18-CAT. The final vector was obtained by replacing the PmeI-AatII fragment of pXI12-ZYIB-EINV4MUTRBS2C with the EcoRI-AatII fragment of pUC18-CAT, carrying the chloramphenicol resistance gene. Figure 23 summarizes the different steps to obtain aforementioned constructs. Both plasmids were transformed into *B. subtilis* strain 1012, and transformants resulting from a Campbell-type integration were selected. Two strains BS1012::SFCONEO1 and BS1012::SFCOCAT1 were chosen for further amplification. Individual colonies of both strains were independently amplified by growing them in different concentrations of antibiotics as described in the methods section. For the cat gene carrying strain, the chloramphenicol concentrations were 60, 80, 120 and 150 mg/ml. For the neo gene carrying strain, the neomycin concentrations were 160 and 180 mg/ml. In both strains only strains with minor amplifications of the SFCO's were obtained. In daughter strains generated from strain BS1012::SFCONEO1, the resistance to higher neomycin concentrations correlated with the increase in the number of SFCO's in the chromosome and with higher levels of carotenoids produced by these cells. A different result was obtained with daughter strains obtained from strain BS1012::SFCOCAT1. In these strains an increase up to 150 mg chloramphenicol/ml resulted, as expected, in a higher number of SFCO copies in the chromosome.

### Example 7

#### C. nstruction of CrtW containing plasmids and use for carotenoid production

*Polymerase chain reaction based gene synthesis.* The nucleotide sequence of the artificial crtW gene, encoding the  $\beta$ -carotene  $\beta$ -4-oxygenase of *Alcaligenes* strain PC-1, was obtained by back translating the amino acid sequence outlined in [Misawa, 1995], using the BackTranslate program of the GCG Wisconsin Sequence Analysis Package, Version 8.0 (Genetics Computer Group, Madison, WI, USA) and a codon frequency reference table of *E. coli* (supplied by the Bach Translate Program). The synthetic gene consisting of 726 nucleotides was constructed basically according to the method described by [Ye, 1992]. The sequence of the 12 oligonucleotides (crtW1 - crtW12) required for the synthesis are shown in Figure 25. Briefly, the long oligonucleotides were designed to have short overlaps of 15-20 bases, serving as primers for the extension of the oligonucleotides. After four cycles a few copies of the full length gene should be present which is then amplified by the two terminal oligonucleotides crtW15 and crtW26. The sequences for these two short oligonucleotides are for the forward primer crtW15 (5'-TATATCTAGAcataTgTCCGGTCGTAAA CCGG-3') and for the reverse primer crtW26 (5'-TATAGaattccacgtgTCA AGCACGACCACCGGTTTTAC G-3'), where the sequences matching the DNA templates are underlined. Small cap letters show the introduced restriction sites (*Nde*I for the forward primer and *Eco*RI and *Pm*II for the reverse primer) for the latter cloning into the pALTER-Ex2 expression vector.

*Polymerase chain reaction.* All twelve long oligonucleotides (crtW1-crtW12; 7 nM each) and both terminal primers (crtW15 and crtW26; 0.1 mM each) were mixed and added to a PCR reaction mix containing Expand™ High Fidelity polymerase (Boehringer, Mannheim) (3.5 units) and dNTP's (100 mM each). The PCR reaction was run for 30 cycles

with the following profile: 94 °C for 1 min, 50 °C for 2 min and 72 °C for 3 min. The PCR reaction was separated on a 1% agarose gel, and the band of approx. 700 bp was excised and purified using the glass beads method (Geneclean Kit, Bio101, Vista, CA, USA). The fragment was subsequently cloned into the *Sma*I site of plasmid pUC18, using the Sure-Clone Kit (Pharmacia, Uppsala, Sweden). The sequence of the resulting crtW synthetic gene was verified by sequencing with the Sequenase Kit Version 1.0 (United States Biochemical, Cleveland, OH, USA). The crtW gene constructed by this method was found to contain minor errors, which were subsequently corrected by site-directed mutagenesis.

**Construction of plasmids.** Plasmid pBIIKS(+)-CARVEG-E (see also Example 5) (Figure 26) contains the carotenoid biosynthesis genes (crtE, crtB, crtY, crtI and crtZ) of the Gram (-) bacterium *Flavobacterium* sp. strain R1534 WT (ATCC 21588) [Pasamontes, 1995 #732] cloned into a modified pBluescript II KS(+) vector (Stratagene, La Jolla, USA) carrying site I of the *B. subtilis* veg promoter [LeGrice, 1986 #806]. This constitutive promoter has been shown to be functional in *E. coli*. Transformants of *E. coli* strain TG1 carrying plasmid pBIIKS(+)-CARVEG-E synthesise zeaxanthin. Plasmid pALTER-Ex2-crtW was constructed by cloning the *Nde*I - *Eco*RI restricted fragment of the synthetic crtW gene into the corresponding sites of plasmid pALTER-Ex2 (Promega, Madison, WI). Plasmid pALTER-Ex2 is a low copy plasmid with the p15a origin of replication, which allows it to be maintained with ColE1 vectors in the same host. Plasmid pBIIKS-crtEBIYZW (Figure 26) was obtained by cloning the *Hind*III-*Pml*I fragment of pALTER-Ex2-crtW into the *Hind*III and the blunt end made *Mlu*I site obtained by a fill in reaction with Klenow enzyme, as described elsewhere in [Sambrook, 1989 #505]. Inactivation of the crtZ gene was done, by deleting a 285 bp *Nsi*I-*Nsi*I fragment, followed by a fill in reaction and religation, resulting in plasmid pBIIKS-crtEBIY[DZ]W. Plasmid pBIIKS-crtEBIY[DZW] carrying the non-functional genes crtW and crtZ, was constructed by digesting the plasmid pBIIKS-crtEBIY[DZ]W with *Nde*I and *Hpa*I, and subsequent self religation of the plasmid after filling in the sites with Klenow enzyme. *E. coli* transformed with this plasmid had a yellow-orange colour due to the accumulation of  $\beta$ -carotene. Plasmid pBIIKS-crtEBIYZ[DW] has a truncated crtW gene obtained by deleting the *Nde*I - *Hpa*I fragment in plasmid pBIIKS-crtEBIYZW as outlined above. Plasmids pALTER-Ex2-crtEBIY[DZW] and pALTER-Ex2-crtEBIYZ[DW], were obtained by isolating the *Bam*HI-*Xba*I fragment from pBIIKS-crtEBIY[DZW] and pBIIKS-crtEBIYZ[DW], respectively and cloning them into the *Bam*HI and *Xba*I sites of pALTER-Ex2. The plasmid pBIIKS-crtW was constructed by digesting pBIIKS-crtEBIYZW with *Nsi*I and *Sac*I, and self-religating the plasmid after recessing the DNA overhangs with Klenow enzyme. Figure 27 compiles the relevant inserts of all the plasmids used in this paper.

**Carotenoid analysis.** *E. coli* TG-1 transformants carrying the different plasmid constructs were grown for 20 hours in Luria-Broth medium supplemented with antibiotics (ampicillin 100 mg/ml, tetracyclin 12.5 mg/ml) in shake flasks at 37°C and 220 rpm. Carotenoids were extracted from the cells with acetone. The acetone was removed in vacuo and the residue was re dissolved in toluene. The coloured solutions were subjected to high-performance liquid chromatography (HPLC) analysis which was performed on a Hewlett-Packard series 1050 instrument. The carotenoids were separated on a silica column Nucleosil Si - 100, 200 x 4 mm, 3m. The solvent system included two solvents: hexane (A) and hexane/THF, 1:1 (B). A linear gradient was applied running from 13 to 50 % (B) within 15 minutes. The flow rate was 1.5 ml/min. Peaks were detected at 450 nm by a photo diode array detector. The individual carotenoid pigments were identified by their absorption spectra and typical retention times as compared to reference samples of chemically pure carotenoids, prepared by chemical synthesis and characterised by NMR, MS and UV-Spectra. HPLC analysis of the pigments isolated from *E. coli* cells transformed with plasmid pBIIKS-crtEBIYZW, carrying besides the carotenoid biosynthesis genes of *Flavobacterium* sp. strain R1534, also the crtW gene encoding the  $\beta$ -carotene ketolase of *Alcaligenes* PC-1 [Misawa, 1995 #670] gave the following major peaks identified as: b-cryptoxanthin, astaxanthin, adonixanthin and zeaxanthin, based on the retention times and on the comparison of the absorbance spectra to given reference samples of chemically pure carotenoids. The relative amount (area percent) of the accumulated pigment in the *E. coli* transformant carrying pBIIKS-crtEBIYZW is shown in Table 3 ["CRX": cryptoxanthin; "ASX": astaxanthin; "ADX": adonixanthin; "ZXX": zeaxanthin; "ECM": echinenone; "MECH": 3-hydroxyechinenone, "CXN": cantaxanthin]. The  $\Sigma$  of the peak areas of all identified carotenoids was defined as 100%. Numbers shown in Table 3 represent the average value of four independent cultures for each transformant. In contrast to the aforementioned results, *E. coli* transformants carrying the same genes but on two plasmids namely, pBIIKS-crtEBIYZ[DW] and pALTER-Ex2-crtW, showed a drastical drop in adonixanthin and a complete lack of astaxanthin pigments (Table 3), whereas the relative amount of zeaxanthin (%) had increased. Echinenone, hydroxyechinenone and canthaxanthin levels remained unchanged compared to the transformants carrying all the crt genes on the same plasmid (pBIIKS-crtEBIYZDW). Plasmid pBIIKS-crtEBIYZ[DW] is a high copy plasmid carrying the functional genes of crtE, crtB, crtY, crtI, crtZ of *Flavobacterium* sp. strain R1534 and a truncated, non-functional version of the crtW gene, whereas the functional copy of the crtW gene is located on the low copy plasmid pALTER-Ex2-crtW. To analyze the effect of overexpression of the crtW gene with respect to the crtZ gene, *E. coli* cells were co-transformed with plasmid pBIIKS-crtW carrying the crtW gene on the high copy plasmid pBIIKS-crtW and the low copy construct pALTER-Ex2-crtEBIYZ[DW], encoding the *Flavobacterium* crt genes. Pigment analysis of these transformants by HPLC monitored the presence of  $\beta$ -carotene, cryptoxanthin, astaxanthin, adonixanthin, zeaxanthin, 3-hydroxyechinenone and minute traces of echinenone and canthaxanthin (Table 3).

Transformants harbouring the crtW gene on the low copy plasmid pALTER-Ex2-crtW and the genes crtE, crtB, crtY and crtI on the high copy plasmid pBIIKS-crtEBIY[DZW] expressed only minor amounts of canthaxanthin (6 %) but high levels of echinenone (94%), whereas cells carrying the crtW gene on the high copy plasmid pBIIKS-crtW and the other crt genes on the low copy construct pALTER-Ex2-crtEBIY[DZW], had 78.6 % and 21.4 % of echinenone and canthaxanthin, respectively (Table 3).

Table 3

plasmids	CRX	ASX	ADX	ZXN	ECH	HECH	CXN
pBIIKS-crtEBIYZW	1.1	2.0	44.2	52.4	< 1	< 1	< 1
pBIIKS-crtEBIYZ[ΔW] + pALTER-Ex2-crtW	2.2	-	25.4	72.4	< 1	< 1	< 1
pBIIKS-crtEBIY[ΔZ]W	-	-	-	-	66.5	-	33.5
pBIIKS-crtEBIY[ΔZW] + pBIIKS-crtW	-	-	-	-	94	-	6

### Example 8

#### Selective carotenoid production by using the crtW and crtZ genes of the Gram negative bacterium E-396.

In this section we describe *E. coli* transformants which accumulate only one (canthaxanthin) or two main carotenoids (astaxanthin, adonixanthin) and minor amounts of adonirubin, rather than the complex variety of carotenoids seen in most carotenoid producing bacteria [Yokoyama et al., Biosci. Biotechnol. Biochem. 58:1842-1844 (1994)] and some of the *E. coli* transformants shown in Table 3. The ability to construct strains producing only one carotenoid is a major step towards a successful biotechnological carotenoid production process. This increase in the accumulation of individual carotenoids accompanied by a decrease of the intermediates, was obtained by replacing the crtZ of *Flavobacterium* R1534 and/or the synthetic crtW gene (see example 5) by their homologous genes originating from the astaxanthin producing Gram negative bacterium E-396 (FERM BP-4283) [Tsubokura et al., EP-application 0 635 576 A1]. Both genes, crtW<sub>E396</sub> and crtZ<sub>E396</sub>, were isolated and used to construct new plasmids as outlined below.

*Isolation of a putative fragment of the crtW gene of strain E-396 by the polymerase chain reaction.* Based on protein sequence comparison of the crtW enzymes of *Agrobacterium aurantiacum*, *Alcaligenes PC-1* (WO95/18220) [Misaawa et al., J.Bacteriol. 177: 6575-6584 (1995)] and *Haematococcus pluvialis* [Kajiwarra et al., Plant Mol. Biol. 29:343-352 (1995)] [Lotan et al., FEBS letters, 364:125-128 (1995)], two regions named I and II, having high amino acid conservation and located approx. 140 amino acids apart, were identified and chosen to design the degenerate PCR primers shown below. The N-terminal peptide HDAMHG (region I) was used to design the two 17-mer degenerate primer sequences crtW100 and crtW101:

crtW100: 5'-CA(C/T)GA(C/T)GC(A/C)ATGCA(C/T)GG-3'

crtW101: 5'-CA(C/T)GA(C/T)GC(G/T)ATGCA(C/T)GG-3'

The C-terminal peptide H(W/H)EHH(R/L) corresponding to region II was used design the two 17-mer degenerate primer with the antisense sequences crtW105 and crtW106:

crtW105: 5'-AG(G/A)TG(G/A)TG(T/C)TC(G/A)TG(G/A)TG-3'

crtW106: 5'-AG(G/A)TG(G/A)TG(T/C)TCCCA(G/A)TG-3'

*Polymerase chain reaction.* PCR was performed using the GeneAmp Kit (Perkin Elmer Cetus) according to the manufacturer's instructions. The different PCR reactions contained combinations of the degenerate primers (crtW100/crtW105 or crtW100/crtW106 or crtW101/crtW105 or crtW101/crtW106) at a final concentration of 50 pM each, together with genomic DNA of the bacterium E-396 (200 ng) and 2.5 units of Taq polymerase. In total 35 cycles of PCR were performed with the following cycle profile: 95 °C for 30 sec, 55 °C for 30 sec, 72 °C for 30 sec. PCR reactions made with the following primer combinations crtW100/crtW105 and crtW101/crtW105 gave PCR amplification products of approx. 500 bp which were in accordance with the expected fragment size. The 500 bp fragment, JAPclone8, obtained in the PCR reaction using primers crtW101 and crtW105 was excised from an 1.5% agarose gel and purified using the GENECLEAN Kit and subsequently cloned into the SmaI site of pUC18 using the Sure-Clone Kit,

according to the manufacturer's instructions. The resulting plasmid was named pUC18-JAPclone8 and the insert was sequenced. Comparison of the determined sequence to the crtW gene of *Agrobacterium aurantiacum* (GenBank accession n° D58420) published by Misawa *et al.* in 1995 (WO95/18220) showed 96% identity at the nucleotide sequence level, indicating that both organisms might be closely related.

5 *Isolation of the crt cluster of the strain E-396.* Genomic DNA of E-396 was digested overnight with different combinations of restrictions enzymes and separated by agarose gel electrophoresis before transferring the resulting fragments by Southern blotting onto a nitrocellulose membrane. The blot was hybridised with a <sup>32</sup>P labelled 334 bp fragment obtained by digesting the aforementioned PCR fragment JAPclone8 with *Bss*HI and *Mlu*I. An approx. 9.4kb *Eco*RI/*Bam*HI fragment hybridizing to the probe was identified as the most appropriate for cloning since it is long enough  
10 to potentially carry the complete crt cluster. The fragment was isolated and cloned into the *Eco*RI and *Bam*HI sites of pBluescriptIIKS resulting in plasmid pJAPCL544 (Fig. 29). Based on the sequence of the PCR fragment JAPclone8, two primers were synthesized to obtain more sequence information left and right hand of this fragment. Fig. 30 shows the sequence obtained containing the crtW<sub>E396</sub> (from nucleotide 40 to 768) and crtZ<sub>E396</sub> (from nucleotide 765 to 1253) genes of the bacterium E-396. The nucleotide sequence of the crtW<sub>E396</sub> gene is shown in Fig. 31 and the encoded amino acid sequence in Fig. 32. The nucleotide sequence of the crtZ<sub>E396</sub> gene is shown in Fig. 33 and the corresponding amino acid sequence in Fig. 34. Comparison to the crtW<sub>E396</sub> gene of E-396 to the crtW gene of *A. aurantiacum* showed 97 % identity at the nucleotide level and 99 % identity at the amino acid level. For the crtZ gene the values were 98 % and 99 %, respectively.

*Construction of plasmids:* Both genes, crtW<sub>E396</sub> and crtZ<sub>E396</sub>, which are adjacent in the genome of E-393, were  
20 isolated by PCR using primer crtW107 and crtW108 and the Expand<sup>TM</sup> High Fidelity PCR system of Boehringer Mannheim, according to the manufacturer's recommendations. To facilitate the subsequent cloning steps (see section below) the primer crt107 (5'-ATCATATGAGCGCACATGCCCTGCCAAGGC-3') contains an artificial *Nde*I site (underlined sequence) spanning the ATG start codon of the crtW<sub>E396</sub> gene and the reverse primer crtW108 (5'-ATCTCGAGT-CACGTGCGCTCCTGCGCCTCGGCC-3') has an *Xho*I site (underlined sequence) just downstream of the TGA stop  
25 codon of the crtZ<sub>E396</sub> gene. The final PCR reaction mix had 10 pM of each primer, 2.5 mg genomic DNA of the bacterium E-396 and 3.5 units of the TaqDNA/Pwo DNA polymerase mix. In total 35 cycles were performed with the following cycle profile: 95 °C, 1 min; 60 °C, 1 min; 72 °C 1min 30 sec. The PCR product of approx. 1250bp was isolated from the 1% agarose gel and purified using GENECLAN before ligation into the *Sma*I site pUC18 using the Sure-Clone Kit. The resulting construct was named pUC18-E396crtWZPCR (Fig. 35). The functionality of both genes was tested as follows. The crtW<sub>E396</sub> and crtZ<sub>E396</sub> gene were isolated from plasmid pUC18-E396crtWZPCR with *Nde*I and *Xho*I and  
30 cloned into the *Nde*I and *Sa*I site of plasmid pBIISK-crtEBIYZW resulting in plasmid pBIISK-crtEBIY[E396WZ] (Fig. 36). *E. coli* TG1 cells transformed with this plasmid produced astaxanthin, adonixanthin and adonirubin but no zeaxanthin (Table 4).

Plasmid pBIISK-crtEBIY[E396W]DZ has a truncated non-functional crtZ gene. Fig. 37 outlines the construction of  
35 this plasmid. The PCR reaction was run as outlined elsewhere in the text using primers crtW113/crtW114 and 200 ng of plasmid pUC18-JAPclone8 as template using 20 cycles with the following protocol: 95 °C, 45 sec/ 62 °C, 20 sec/ 72 °C, 20 sec)

primer crtW113 (5'-ATATACATATGGTGTCCCCCTTGGTGCGGGTGTC-3')

40

primer crtW114 (5'-TATGGATCCGACGCGTTCCCGGACCGCCACAATGC-3')

The resulting 150 bp fragment was digested with *Bam*HI and *Nde*I and cloned into the corresponding sites of pBIISK(+)-PCRRBSrtZ resulting in the construct pBIISK(+)-PCRRBSrtZ-2. The final plasmid carrying the genes crtE, crtB, crtI, crtY of *Flavobacterium*, the crtW<sub>E396</sub> gene of E-396 and a truncated non-functional crtZ gene of  
45 *Flavobacterium* was obtained by isolating the *Mlu*I/*Nru*I fragment (280 bp) of pBIISK(+)-PCRRBSrtZ-2 and cloning it, into the *Mlu*I/*Pml*I sites of plasmid pBIISK-crtEBIY[E396WZ]. *E. coli* cells transformed with this plasmid produced 100% canthaxanthin (Table 4; "CRX": cryptoxanthin; "ASX": astaxanthin; "ADX": adonixanthin; "ZXN": zeaxanthin; "ECH": echinenone; "HECH": 3-hydroxyechinenone; "CXN": canthaxanthin; "BCA": β-carotene; "ADR": adonirubin; Numbers indicate the % of the individual carotenoid of the total carotenoids produced in the cell.).  
50

Table 4

55	plasmid	CRX	ASX	ADX	ZXN	ECH	HECH	CXN	BCA	ADR
	pBIISKrtEBIYZW	1.1	2.0	44.2	52.4	<1	<1	<1		
	pBIISK-crtEBIY[E396WZ]		74.4	19.8						5.8

Table 4 (continued)

plasmid	CRX	ASX	ADX	ZXN	ECH	HECH	CXN	BCA	ADR
pBIIKS-crtEBIY[E396W] $\Delta$ Z							100		

The results of *E. coli* transformants carrying pBIIKScrtEBIYZW (see example 7) are also shown in Table 4 to indicate the dramatic effect of the new genes crtW<sub>E396</sub> and crtZ<sub>E396</sub> on the carotenoids produced in these new transformants.

### Example 9

#### Cloning of the remaining crt genes of the Gram negative bacterium E-396.

TG1 *E. coli* transformants carrying the pJAPCL544 plasmid did not produce detectable quantities of carotenoids (results not shown). Sequence analysis and comparison of the 3' (*Bam*HI site) of the insert of plasmid pJAPCL544, to the crt cluster of *Flavobacterium* R1534 showed that only part of the C-terminus of the crtE gene was present. This result explained the lack of carotenoid production in the aforementioned transformants. To isolate the missing N-terminal part of the gene, genomic DNA of E-396 was digested by 6 restrictions enzymes in different combinations : *Eco*RI, *Bam*HI, *Pst*I, *Sac*I, *Sph*I and *Xba*I and transferred by the Southern blot technique to nitrocellulose. Hybridization of this membrane with the <sup>32</sup>P radio-labelled probe (a 463 bp *Pst*I-*Bam*HI fragment originating from the 3' end of the insert of pJAPCL544 (Fig. 29) highlighted a ~1300 bp-long *Pst*I-*Pst*I fragment. This fragment was isolated and cloned into the *Pst*I site of pBSIIKS(+) resulting in plasmid pBSIIKS-#1296. The sequence of the insert is shown in Fig. 38 (small cap letters refer to new sequence obtained. Capital letters show the sequence also present in the 3' of the insert of plasmid pJAPCL544). The complete crtE gene has therefore a length of 882 bp (see Fig. 39) and encodes a GGPP synthase of 294 amino acids (Fig. 40). The crtE enzyme has 38 % identity with the crtE amino acid sequence of *Erwinia herbicola* and 66 % with *Flavobacterium* R1534 WT.

**Construction of plasmids.** To have a plasmid carrying the complete crt cluster of E-396, the 4.7 kb *Mlu*I/*Bam*HI fragment encoding the genes crtW, crtZ, crtY, crtI and crtB was isolated from pJAPCL544 and cloned into the *Mlu*I/*Bam*HI sites of pUC18-E396crtWZPCR (see example 8). The new construct was named pE396CARcrtW-B (Fig. 41) and lacked the N-terminus of the crtE gene. The missing C-terminal part of the crtE gene was then introduced by ligation of the aforementioned *Pst*I fragment of pBSIIKS-#1296 between the *Pst*I sites of pE396CARcrtW-B. The resulting plasmid was named pE396CARcrtW-E (Fig. 41). The carotenoid distribution of the *E. coli* transformants carrying aforementioned plasmid were: adonixanthin (65%), astaxanthin (8%) and zeaxanthin (3%). The % indicated reflects the proportion of the total amount of carotenoid produced in the cell.

### Example 10

#### Astaxanthin and adonixanthin production in *Flavobacterium* R1534

Among bacteria *Flavobacterium* may represent the best source for the development of a fermentative production process for 3R, 3R' zeaxanthin. Derivatives of *Flavobacterium* sp. strain R1534, obtained by classical mutagenesis have attracted in the past two decades wide interest for the development of a large scale fermentative production of zeaxanthin, although with little success. Cloning of the carotenoid biosynthesis genes of this organism, as outlined in example 2, may allow replacement of the classical mutagenesis approach by a more rational one, using molecular tools to amplify the copy number of relevant genes, deregulate their expression and eliminate bottlenecks in the carotenoid biosynthesis pathway. Furthermore, the introduction of additional heterologous genes (e.g. crtW) will result in the production of carotenoids normally not synthesised by this bacterium (astaxanthin, adonirubin, adonixanthin, canthaxanthin, echinenone). The construction of such recombinant *Flavobacterium* R1534 strains producing astaxanthin and adonixanthin will be outlined below.

#### Gene transfer into *Flavobacterium* sp.

**Plasmid transfer by conjugative mobilization.** For the conjugational crosses we constructed plasmid pRSF1010-Amp<sup>r</sup>, a derivative of the small (8.9 kb) broad host range plasmid RSF1010 (IncQ incompatibility group) [Guerry et al., J. Bacteriol. 117:619-630 (1974)] and used *E. coli* S17-1 as the mobilizing strain [Priefer et al., J. Bacteriol. 163:324-330 (1985)]. In general any of the IncQ plasmids (e.g. RSF1010, R300B, R1162) may be mobilized into rifampicin resistant *Flavobacterium* if the transfer functions are provided by plasmids of the IncP1 group (e.g. R1, R751).

Rifampicin resistant (Rif<sup>r</sup>) *Flavobacterium* R1534 cells were obtained by selection on 100 mg rifampicin/ml. One resistant colony was picked and a stock culture was made. The conjugation protocol was as follows:

## EP 0 872 554 A2

### Day 1:

- grow 3 ml culture of *Flavobacterium* R1534 Rif<sup>r</sup> for 24 hours at 30 °C in Flavobacter medium (F-medium) (see example 1)
- grow 3 ml mobilizing *E. coli* strain carrying the mobilizable plasmid O/N at 37 °C in LB medium. (e.g. *E. coli* S17-1 carrying pRSF1010-Amp<sup>r</sup> or *E. coli* TG-1 cells carrying R751 and pRSF1010-Amp<sup>r</sup>)

### Day 2:

- pellet 1 ml of the *Flavobacterium* R1534 Rif<sup>r</sup> cells and resuspend in 1ml of fresh F-medium.
- pellet 1 ml of *E. coli* cells (see above) and resuspend in 1 ml of LB medium.
- donor and recipient cells are then mixed in a ratio of 1:1 and 1: 10 in an Eppendorf tube and 30 µl are then applied onto a nitrocellulose filter plated on agar plates containing F-medium and incubated O/N at 30°C.

### Day 3:

- the conjugational mixtures were washed off with F-medium and plated on F-medium containing 100 mg rifampicin and 100 mg ampicillin/ml for selection of transconjugants and inhibition of the donor cells.

### Day 6-8

- Arising clones are plated once more on F-medium containing 100 mg Rif and 100 mg Amp/ml before analysis.

Plasmid transfer by electroporation. The protocol for the electroporation is as follows:

1. add 10 ml of O/N culture of *Flavobacterium* sp. R1534 into 500 ml F-medium and incubate at 30°C until OD<sub>600</sub>=0.8-0.1

2. harvest cells by centrifugation at 4000g at 4°C for 10 min.

3. wash cells in equal volume of ice-cold deionized water (2 times)

4. resuspend bacterial pellet in 1 ml ice-cold deionized water

5. take 50 µl of cells for electroporation with 0.1 µg of plasmid DNA

6. electroporation was done using field strengths between 15 and 25 kV/cm and 1-3 ms.

7. after electroporation cells were immediately diluted in 1 ml of F-medium and incubated for 2 hours at 30°C at 180 rpm before plating on F-medium plates containing the respective selective antibiotic.

Plasmid constructions: Plasmid pRSF101-Amp<sup>r</sup> was obtained by cloning the Amp<sup>r</sup> gene of pBR322 between the *EcoRI*/*NotI* sites of RSF1010. The Amp<sup>r</sup> gene originates from pBR322 and was isolated by PCR using primers AmpR1 and AmpR2 as shown in Fig. 42.

AmpR1:

5'-TATATCGGCCGACTAGTAAGCTTCAAAAAGGATCTTCACCTAG-3' the underlined sequence contains the introduced restriction sites for *EagI*, *SpeI* and *HindIII* to facilitate subsequent constructions.

AmpR2:

5'-ATATGAATTCAATAATATTGAAAAAGGAAG-3' the underlined sequence corresponds to an introduced *EcoRI* restriction site to facilitate cloning into RSF1010 (see Fig. 42).

The PCR reaction mix had 10 pM of each primer (AmpR1/AmpR2), 0.5 µg plasmid pBR322 and 3.5 units of the TaqDNA/Pwo DNA polymerase mix. In total 35 amplification cycles were made with the profile: 95 °C, 45 sec; 59 °C, 45 sec, 72 °C, 1 min. The PCR product of approx. 950 was extracted once with phenol/chloroform and precipitated with 0.3

M NaAcetate and 2 vol. Ethanol. The pellet was resuspended in H<sub>2</sub>O and digested with *EcoRI* and *EagI* O/N. The digestion was separated by electrophoresis and the fragment isolated from the 1% agarose gel and purified using GENECLAN before ligation into the *EcoRI* and *NotI* sites of RSF1010. The resulting plasmid was named pRSF1010-Amp<sup>r</sup> (Fig. 42).

Plasmid RSF1010-Amp<sup>r</sup>-crt1 was obtained by isolating the *HindIII/NotI* fragment of pBIKS-crtEBIY[E396WZ] and cloning it between the *HindIII/EagI* sites of RSF1010-Amp<sup>r</sup> (Fig. 43). The resulting plasmid RSF1010-Amp<sup>r</sup>-crt1 carries crtW<sub>E396</sub>, crtZ<sub>E396</sub>, crtY genes and the N-terminus of the crtI gene (non-functional). Plasmid RSF1010-Amp<sup>r</sup>-crt2 carrying a complete crt cluster composed of the genes crtW<sub>E396</sub> and crtZ<sub>E396</sub> of E-396 and the crtY, crtI, crtB and crtE of *Flavobacterium* R1534 was obtained by isolating the large *HindIII/XbaI* fragment of pBIKS-crtEBIY[E396WZ] and cloning it into the *SpeI/HindIII* sites of RSF1010-Amp<sup>r</sup> (Fig. 43).

*Flavobacterium* R1534 transformants carrying either plasmid RSF1010-Amp<sup>r</sup>, Plasmid RSF1010-Amp<sup>r</sup>-crt1 or Plasmid RSF1010-Amp<sup>r</sup>-crt2 were obtained by conjugation as outlined above using *E. coli* S17-1 as mobilizing strain.

Comparison of the carotenoid production of two *Flavobacterium* transformants. Overnight cultures of the individual transformants were diluted into 20 ml fresh F-medium to have a final starting OD<sub>600</sub> of 0.4. Cells were harvested after growing for 48 hours at 30 °C and carotenoid contents were analysed as outlined in example 7. Table 5 shows the result of the three control cultures *Flavobacterium* [R1534 WT], [R1534 WT Rif<sup>r</sup>] (rifampicin resistant) and [R1534WT Rif<sup>r</sup> RSF1010-Amp<sup>r</sup>] (carries the RSF1010-Amp<sup>r</sup> plasmid) and the two transformants [R1534 WT RSF1010-Amp<sup>r</sup>-crt1] and [R1534 WT RSF1010-Amp<sup>r</sup>-crt2]. Both latter transformants are able to synthesise astaxanthin and adonixanthin but little zeaxanthin. Most interesting is the [R1534 WT RSF1010-Amp<sup>r</sup>-crt2] *Flavobacterium* transformant which produces approx. 4 times more carotenoids than the R1534 WT. This increase in total carotenoid production is most likely due to the increase of the number of carotenoid biosynthesis clusters present in these cell (e.g. corresponds to the total copy number of plasmids in the cell).

Table 5

Transformant	carotenoids % of total dry weight	total carotenoid content in % of dry weight
R1534 WT	0.039% β-Carotin 0.001% β-Cryptoxanthin 0.018% Zeaxanthin	0.06%
R1534 Rif <sup>r</sup>	0.036% β-Carotin 0.002% β-Cryptoxanthin 0.022% Zeaxanthin	0.06%
R1534 Rif <sup>r</sup> [RSF1010-Amp <sup>r</sup> ]	0.021% β-Carotin 0.002% β-Cryptoxanthin 0.032% Zeaxanthin	0.065%
R1534 Rif <sup>r</sup> [RSF1010-Amp <sup>r</sup> -crt1]	0.022% Astaxanthin 0.075% Adonixanthin 0.004% Zeaxanthin	0.1%
R1534 Rif <sup>r</sup> [RSF1010-Amp <sup>r</sup> -crt2]	0.132% β-Carotin 0.006% Echinenon 0.004% Hydroxyechinenon 0.003% β-Cryptoxanthin 0.044% Astaxanthin 0.039% Adonixanthin 0.007% Zeaxanthin	0.235%

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

## (i) APPLICANT:

(A) NAME: F.HOFFMANN-LA ROCHE AG  
(B) STREET: GRENZACHERSTRASSE 124  
(C) CITY: BASLE  
(D) STATE: BS  
(E) COUNTRY: SWITZERLAND  
(F) POSTAL CODE (ZIP): CH - 4002  
(G) TELEPHONE: 061 - 688 2505  
(H) TELEFAX: 061 688 1395  
(I) TELEX: 962292/965542 hlr ch

(ii) TITLE OF INVENTION: Improved fermentative carotenoid production

(iii) NUMBER OF SEQUENCES: 17

## (iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

## (v) CURRENT APPLICATION DATA:

APPLICATION NUMBER: EP 97120324.5

## (2) INFORMATION FOR SEQ ID NO: 1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 729 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

35	ATGAGCGCAC ATGCCCTGCC CAAGGCAGAT CTGACCGCCA CCAGTTTGAT CGTCTCGGGC	60
	GGCATCATCG CCGCGTGGCT GGCCCTGCAT GTGCATGCGC TGTGGTTTCT GGACGCGGCG	120
	GCGCATCCCA TCCTGGCGGT CGCGAATTTT CTGGGGCTGA CCTGGCTGTC GGTTCGGTCTG	180
40	TTCATCATCG CGCATGACGC GATGCATGGG TCGGTCGTGC CGGGGCGCCC GCGCGCCAAT	240
	GCGGCGATGG GCCAGCTTGT CCTGTGGCTG TATGCCGGAT TTTCTGGCG CAAGATGATC	300
	GTCAAGCACA TGGCCCATCA TCGCCATGCC GGAACCGACG ACGACCCAGA TTTCGACCAT	360
	GGCGGCCCCG TCCGCTGGTA CGCCCGCTTC ATCGGCACCT ATTTCGGCTG GCGCGAGGGG	420
45	CTGCTGCTGC CCGTCATCGT GACGGTCTAT GCGCTGATGT TGGGGATCG CTGGATGTAC	480
	GTGGTCTTCT GGCCGTTGCC GTCGATCCTG GCGTCGATCC AGCTGTTTCGT GTTCGGCATC	540
	TGGCTGCCGC ACCGCCCCGG CCACGACGCG TTCCCGGACC GCCACAATGC GCGGTCGTGC	600
50	CGGATCAGCG ACCCCGTGTC GCTGCTGACC TGCTTTCACT TTGGCGGTTA TCATCACGAA	660
	CACCACCTGC ACCCGACGGT GCCTTGGTGG CGCCTGCCCA GCACCCGCAC CAAGGGGGAC	720
	ACCGCATGA	729

# EP 0 872 554 A2

## (2) INFORMATION FOR SEQ ID NO: 2:

### (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 242 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

### (ii) MOLECULE TYPE: protein

### (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```

Met Ser Ala His Ala Leu Pro Lys Ala Asp Leu Thr Ala Thr Ser Leu
1           5           10           15
Ile Val Ser Gly Gly Ile Ile Ala Ala Trp Leu Ala Leu His Val His
15          20          25          30
Ala Leu Trp Phe Leu Asp Ala Ala Ala His Pro Ile Leu Ala Val Ala
35          40          45
Asn Phe Leu Gly Leu Thr Trp Leu Ser Val Gly Leu Phe Ile Ile Ala
20          50          55          60
His Asp Ala Met His Gly Ser Val Val Pro Gly Arg Pro Arg Ala Asn
65          70          75          80
Ala Ala Met Gly Gln Leu Val Leu Trp Leu Tyr Ala Gly Phe Ser Trp
25          85          90          95

Arg Lys Met Ile Val Lys His Met Ala His His Arg His Ala Gly Thr
100         105         110
Asp Asp Asp Pro Asp Phe Asp His Gly Gly Pro Val Arg Trp Tyr Ala
115         120         125
Arg Phe Ile Gly Thr Tyr Phe Gly Trp Arg Glu Gly Leu Leu Leu Pro
130         135         140
Val Ile Val Thr Val Tyr Ala Leu Met Leu Gly Asp Arg Trp Met Tyr
145         150         155
Val Val Phe Trp Pro Leu Pro Ser Ile Leu Ala Ser Ile Gln Leu Phe
165         170         175
Val Phe Gly Ile Trp Leu Pro His Arg Pro Gly His Asp Ala Phe Pro
180         185         190
Asp Arg His Asn Ala Arg Ser Ser Arg Ile Ser Asp Pro Val Ser Leu
195         200         205
Leu Thr Cys Phe His Phe Gly Gly Tyr His His Glu His His Leu His
210         215         220
Pro Thr Val Pro Trp Trp Arg Leu Pro Ser Thr Arg Thr Lys Gly Asp
225         230         235         240
Thr Ala

```

## (2) INFORMATION FOR SEQ ID NO: 3:

### (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 486 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

# EP 0 872 554 A2

(ii) MOLECULE TYPE: DNA (genomic)

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

```

ATGACCAATT TCCTGATCGT CGTCGCCACC GTGCTGGTGA TGGAGCTGAC GGCCTATTCC      60
GTCCACCGCT GGATCATGCA CGGCCCTTG GGCTGGGGCT GGCACAAGTC CCACCACGAG      120
GAACACGACC ACGCGCTGGA AAAGAACGAC CTGTACGGCC TGGTCTTTGC GGTGATCGCC      180
10 ACGGTGCTGT TCACGGTGGG CTGGATCTGG GCACCGGTCC TGTGGTGGAT CGCCTTGGGC      240
ATGACCGTCT ACGGGCTGAT CTATTTCTGC CTGCATGACG GGCTGGTGCA TCAGCGCTGG      300
CCGTTCCGCT ATATCCCTCG CAAGGGCTAT GCCAGACGCC TGTATCAGGC CCACCGCCTG      360
15 CACCACGCGG TCGAGGGGCG CGACCATTCG GTCAGCTTCG GCTTCATCTA TGCGCCGCCG      420
GTCGACAAGC TGAAGCAGGA CCTGAAGACG TCGGGCGTGC TCGGGGCCGA GCGCAGGAG      480
CGCACG                                         486

```

20 (2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 162 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

25 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

```

30 Met Thr Asn Phe Leu Ile Val Val Ala Thr Val Leu Val Met Glu Leu
   1           5           10           15
Thr Ala Tyr Ser Val His Arg Trp Ile Met His Gly Pro Leu Gly Trp
           20           25           30
35 Gly Trp His Lys Ser His His Glu Glu His Asp His Ala Leu Glu Lys
   35           40           45
Asn Asp Leu Tyr Gly Leu Val Phe Ala Val Ile Ala Thr Val Leu Phe
   50           55           60
40 Thr Val Gly Trp Ile Trp Ala Pro Val Leu Trp Trp Ile Ala Leu Gly
   65           70           75           80
Met Thr Val Tyr Gly Leu Ile Tyr Phe Val Leu His Asp Gly Leu Val
           85           90           95
His Gln Arg Trp Pro Phe Arg Tyr Ile Pro Arg Lys Gly Tyr Ala Arg
           100          105          110
45 Arg Leu Tyr Gln Ala His Arg Leu His His Ala Val Glu Gly Arg Asp
   115          120          125
His Cys Val Ser Phe Gly Phe Ile Tyr Ala Pro Pro Val Asp Lys Leu
   130          135          140
50 Lys Gln Asp Leu Lys Thr Ser Gly Val Leu Arg Ala Glu Ala Gln Glu
   145          150          155          160
Arg Thr

```

55

# EP 0 872 554 A2

## (2) INFORMATION FOR SEQ ID NO: 5:

### (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 882 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

### (ii) MOLECULE TYPE: DNA (genomic)

### (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

```

ATGAGACGAG ACGTCAACCC GATCCACGCC ACCCTTCTGC AGACCAGACT TGAGGAGATC      60
GCCCAGGGAT TCGGTGCCGT GTCGCAGCCG CTCGGCCCGG CCATGAGCCA TGGCGCGCTG      120
TCGTGCGGCA AGCGTTTCCG CGGCATGCTG ATGTGCTTG CGGCAGAAGC CTCGGGCGGG      180
GTCTGCGACA CGATCGTCGA CGCCGCCTGC GCGGTGCGA TGGTGCATGC CGCATCGCTG      240
ATCTTCGACG ACCTGCCCTG CATGGACGAT GCCGGGCTGC GCCGCGGCCA GCCC GCGACC      300
CATGTGGCGC ATGGCGAAAG CCGCGCCGTG CTAGGCGGCA TCGCCCTGAT CACCGAGGCG      360
ATGGCCCTGC TGGCCGGTGC GCGCGGCGCG TCGGGCACGG TCGGGGCGCA GCTGGTGCGG      420
ATCCTGTGCG GGTCCTTGGG GCCGAGGGC CTGTGCGCCG GCCAGGACCT GGACCTGCAC      480
GCGGCCAAGA ACGGCGCGGG GGTGCAACAG GAACAGGACC TGAAGACCGG CGTGCTGTTC      540
ATCGCCGGGC TGGAGATGCT GGCCGTGATC AAGGAGTTCG ACGCCGAGGA GCAGACTCAG      600
ATGATCGACT TTGGCCGTCA GCTGGGCCGG GTGTTCCAGT CCTATGACGA CCTGCTGGAC      660
GTTGTGGGCG ACCAGGCGGC GCTTGCAAG GATACGGTC GCGATGCGGC GGCCCCGGC      720
CCGCGGCGCG GCCTTCTGGC CGTGTGAGAC CTGCAGAACG TGTCCCGTCA CTATGAGGCC      780
AGCCGCGCCC AGCTGGACGC GATGTGCGC AGCAAGCGCC TTCAGGCTCC GGAAATCGCG      840
GCCCTGCTGG AACGGTTCT GCCCTACGCC GCGCGCGCCT AG                          882

```

## (2) INFORMATION FOR SEQ ID NO: 6:

### (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 293 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

### (ii) MOLECULE TYPE: protein

### (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

```

Met Arg Arg Asp Val Asn Pro Ile His Ala Thr Leu Leu Gln Thr Arg      15
 1           5           10
Leu Glu Glu Ile Ala Gln Gly Phe Gly Ala Val Ser Gln Pro Leu Gly      30
 20           25           30
Pro Ala Met Ser His Gly Ala Leu Ser Ser Gly Lys Arg Phe Arg Gly      45
 35           40           45
Met Leu Met Leu Leu Ala Ala Glu Ala Ser Gly Gly Val Cys Asp Thr

```

# EP 0 872 554 A2

50 55 60

Ile Val Asp Ala Ala Cys Ala Val Glu Met Val His Ala Ala Ser Leu  
65 70 75 80

Ile Phe Asp Asp Leu Pro Cys Met Asp Asp Ala Gly Leu Arg Arg Gly  
85 90 95

Gln Pro Ala Thr His Val Ala His Gly Glu Ser Arg Ala Val Leu Gly  
100 105 110

Gly Ile Ala Leu Ile Thr Glu Ala Met Ala Leu Leu Ala Gly Ala Arg  
115 120 125

Gly Ala Ser Gly Thr Val Arg Ala Gln Leu Val Arg Ile Leu Ser Arg  
130 135 140

Ser Leu Gly Pro Gln Gly Leu Cys Ala Gly Gln Asp Leu Asp Leu His  
145 150 155 160

Ala Ala Lys Asn Gly Ala Gly Val Glu Gln Glu Gln Asp Leu Lys Thr  
165 170 175

Gly Val Leu Phe Ile Ala Gly Leu Glu Met Leu Ala Val Ile Lys Glu  
180 185 190

Phe Asp Ala Glu Glu Gln Thr Gln Met Ile Asp Phe Gly Arg Gln Leu  
195 200 205

Gly Arg Val Phe Gln Ser Tyr Asp Asp Leu Leu Asp Val Val Gly Asp  
210 215 220

Gln Ala Ala Leu Gly Lys Asp Thr Gly Arg Asp Ala Ala Ala Pro Gly  
225 230 235 240

Pro Arg Arg Gly Leu Leu Ala Val Ser Asp Leu Gln Asn Val Ser Arg  
245 250 255

His Tyr Glu Ala Ser Arg Ala Gln Leu Asp Ala Met Leu Arg Ser Lys  
260 265 270

Arg Leu Gln Ala Pro Glu Ile Ala Ala Leu Leu Glu Arg Val Leu Pro  
275 280 285

Tyr Ala Ala Arg Ala  
290

## (2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 295 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Met Thr Pro Lys Gln Gln Phe Pro Leu Arg Asp Leu Val Glu Ile Arg  
1 5 10 15

Leu Ala Gln Ile Ser Gly Gln Phe Gly Val Val Ser Ala Pro Leu Gly  
20 25 30

Ala Ala Met Ser Asp Ala Ala Leu Ser Pro Gly Lys Arg Phe Arg Ala  
35 40 45

# EP 0 872 554 A2

Val Leu Met Leu Met Val Ala Glu Ser Ser Gly Gly Val Cys Asp Ala  
 50 55 60  
 Met Val Asp Ala Ala Cys Ala Val Glu Met Val His Ala Ala Ser Leu  
 5 65 70 75 80  
 Ile Phe Asp Asp Met Pro Cys Met Asp Asp Ala Arg Thr Arg Arg Gly  
 85 90 95  
 Gln Pro Ala Thr His Val Ala His Gly Glu Gly Arg Ala Val Leu Ala  
 10 100 105 110  
 Gly Ile Ala Leu Ile Thr Glu Ala Met Arg Ile Leu Gly Glu Ala Arg  
 115 120 125  
 Gly Ala Thr Pro Asp Gln Arg Ala Arg Leu Val Ala Ser Met Ser Arg  
 130 135 140  
 Ala Met Gly Pro Val Gly Leu Cys Ala Gly Gln Asp Leu Asp Leu His  
 15 145 150 155 160  
 Ala Pro Lys Asp Ala Ala Gly Ile Glu Arg Glu Gln Asp Leu Lys Thr  
 165 170 175  
 Gly Val Leu Phe Val Ala Gly Leu Glu Met Leu Ser Ile Ile Lys Gly  
 20 180 185 190  
 Leu Asp Lys Ala Glu Thr Glu Gln Leu Met Ala Phe Gly Arg Gln Leu  
 195 200 205  
 Gly Arg Val Phe Gln Ser Tyr Asp Asp Leu Leu Asp Val Ile Gly Asp  
 210 215 220  
 Lys Ala Ser Thr Gly Lys Asp Thr Ala Arg Asp Thr Ala Ala Pro Gly  
 25 225 230 235 240  
 Pro Lys Gly Gly Leu Met Ala Val Gly Gln Met Gly Asp Val Ala Gln  
 245 250 255  
 His Tyr Arg Ala Ser Arg Ala Gln Leu Asp Glu Leu Met Arg Thr Arg  
 30 260 265 270  
 Leu Phe Arg Gly Gly Gln Ile Ala Asp Leu Leu Ala Arg Val Leu Pro  
 275 280 285  
 His Asp Ile Arg Arg Ser Ala  
 290 295

## (2) INFORMATION FOR SEQ ID NO: 8:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 888 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

45 ATGACGCCCA AGCAGCAATT CCCCTACGC GATCTGGTCG AGATCAGGCT GGCGCAGATC 60  
 TCGGGCCAGT TCGGCGTGGT CTCGGCCCCG CTCGGCGCGG CCATGAGCGA TGCCGCCCTG 120  
 TCCCCGGCA AACGCTTTCG CGCCGTGCTG ATGCTGATGG TCGCCGAAAG CTCGGGCGGG 180  
 50 GTCTGCGATG CGATGGTCGA TGCCGCCTGC GCGGTCGAGA TGGTCCATGC CGCATCGCTG 240  
 ATCTTCGACG ACATGCCCTG CATGGACGAT GCCAGGACCC GTCGCGGTCA GCCCGCCACC 300

# EP 0 872 554 A2

CATGTCGCCC ATGGCGAGGG GCGCGCGGTG CTTGCGGGCA TCGCCCTGAT CACCGAGGCC 360  
 ATGCGGATTT TGGGCGAGGC GCGCGGCGCG ACGCCGGATC AGCGCGCAAG GCTGGTCGCA 420  
 5 TCCATGTCGC GCGCGATGGG ACCGGTGGGG CTGTGCGCAG GGCAGGATCT GGACCTGCAC 480  
 GCCCCCAAGG ACGCCGCCGG GATCGAACGT GAACAGGACC TCAAGACCGG CGTGCTGTTC 540  
 GTCGCGGGCC TCGAGATGCT GTCCATTATT AAGGGTCTGG ACAAGGCCGA GACCGAGCAG 600  
 10 CTCATGGCCT TCGGGCGTCA GCTTGGTTCG GTCTTCCAGT CCTATGACGA CCTGCTGGAC 660  
 GTGATCGGCG ACAAGGCCAG CACCGGAAG GATACGGCGC GCGACACCGC CGCCCCGGC 720  
 CCAAGGGCG CCTCATGCC CCGCGACAG ATGGCGCAG TGGCGCAGCA TTACCGCGCC 780  
 AGCCGCGCGC AACTGGACGA GCTGATGCGC ACCCGGCTGT TCCGCGGGGG GCAGATCGCG 840  
 15 GACCTGCTGG CCCGCTGCT GCCGCATGAC ATCCGCCGCA GCGCCTAG 888

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 303 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

Met Thr Asp Leu Thr Ala Thr Ser Glu Ala Ala Ile Ala Gln Gly Ser  
 1 5 10 15  
 30 Gln Ser Phe Ala Gln Ala Ala Lys Leu Met Pro Pro Gly Ile Arg Glu  
 20 25 30  
 Asp Thr Val Met Leu Tyr Ala Trp Cys Arg His Ala Asp Asp Val Ile  
 35 40 45  
 40 Asp Gly Gln Val Met Gly Ser Ala Pro Glu Ala Gly Gly Asp Pro Gln  
 50 55 60  
 Ala Arg Leu Gly Ala Leu Arg Ala Asp Thr Leu Ala Ala Leu His Glu  
 65 70 75 80  
 Asp Gly Pro Met Ser Pro Pro Phe Ala Ala Leu Arg Gln Val Ala Arg  
 85 90 95  
 40 Arg His Asp Phe Pro Asp Leu Trp Pro Met Asp Leu Ile Glu Gly Phe  
 100 105 110  
 Ala Met Asp Val Ala Asp Arg Glu Tyr Arg Ser Leu Asp Asp Val Leu  
 115 120 125  
 45 Glu Tyr Ser Tyr His Val Ala Gly Val Val Gly Val Met Met Ala Arg  
 130 135 140  
 Val Met Gly Val Gln Asp Asp Ala Val Leu Asp Arg Ala Cys Asp Leu  
 145 150 155 160  
 50 Gly Leu Ala Phe Gln Leu Thr Asn Ile Ala Arg Asp Val Ile Asp Asp  
 165 170 175  
 Ala Ala Ile Gly Arg Cys Tyr Leu Pro Ala Asp Trp Leu Ala Glu Ala  
 180 185 190

# EP 0 872 554 A2

Gly Ala Thr Val Glu Gly Pro Val Pro Ser Asp Ala Leu Tyr Ser Val  
195 200 205  
5 Ile Ile Arg Leu Leu Asp Ala Ala Glu Pro Tyr Tyr Ala Ser Ala Arg  
210 215 220  
Gln Gly Leu Pro His Leu Pro Pro Arg Cys Ala Trp Ser Ile Ala Ala  
225 230 235 240  
10 Ala Leu Arg Ile Tyr Arg Ala Ile Gly Thr Arg Ile Arg Gln Gly Gly  
245 250 255  
Pro Glu Ala Tyr Arg Gln Arg Ile Ser Thr Ser Lys Ala Ala Lys Ile  
260 265 270  
15 Gly Leu Leu Ala Arg Gly Gly Leu Asp Ala Ala Ala Ser Arg Leu Arg  
275 280 285  
Gly Gly Glu Ile Ser Arg Asp Gly Leu Trp Thr Arg Pro Arg Ala  
290 295 300

## (2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 908 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

ATGACCGATC	TGACGGCGAC	TTCCGAAGCG	GCCATCGCGC	AGGGTTCGCA	AAGCTTCGCG	60
CAGGCGGCCA	AGCTGATGCC	GCCCCGCATC	CGCGAGGATA	CGGTCATGCT	CTATGCCTGG	120
TGCAGGCATG	CGGATGACGT	GATCGACGGG	CAGGTGATGG	GTTCTGCCCC	CGAGGCGGGC	180
GGCGACCCAC	AGGCGCGGCT	GGGGGCGCTG	CGCGCCGACA	CGCTGGCCGC	GCTGCACGAG	240
GACGGCCCCA	TGTCGCCGCC	CTTCGCGGCG	CTGCGCCAGG	TCGCCCGGCG	GCATGATTTT	300
CCGGACCTTT	GGCCGATGGA	CCTGATCGAG	GGTTTCGCGA	TGGATGTCGC	GGATCGCGAA	360
TACCGCAGCC	TGGATGACGT	GCTGGAATAT	TCCTACCACG	TCGCGGGGGT	CGTGGGCGTG	420
ATGATGGCGC	GGGTGATGGG	CGTGCAGGAC	GATGCGGTGC	TGGATCGCGC	CTGCGATCTG	480
GGCCTTGCGT	TCCAGCTGAC	GAACATCGCT	CGCGACGTGA	TCGACGATGC	CGCCATCGGG	540
CGCTGCTATC	TGCCTGCCGA	CTGGCTGGCC	GAGGCGGGGG	CGACGGTTGA	GGGTCCGGTG	600
CCTTCGGACG	CGCTCTATT	CGTCATCATC	CGCCTGCTTG	ACGCGGCCGA	GCCCTATTAT	660
GCCTCGGCGC	GGCAGGGGCT	TCCGCATCTG	CCGCCGCGCT	GCGCGTGGTC	GATCGCCGCC	720
GCGCTGCGTA	TCTATCGCGC	AATCGGGACG	CGCATCCGGC	AGGGTGGCCC	CGAGGCCTAT	780
CGCCAGCGGA	TCAGCACGTC	GAAGGCTGCC	AAGATCGGGC	TTCTGGCGCG	CGGAGGCTTG	840
GACGCGGCCG	CATCGCGCCT	GCGCGGCGGC	GAAATCAGCC	GCGACGGCCT	GTGGACCCGA	900
CCGCGCGC						908

# EP 0 872 554 A2

## (2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 494 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

```

Met Ser Ser Ala Ile Val Ile Gly Ala Gly Phe Gly Gly Leu Ala Leu
1      5      10
Ala Ile Arg Leu Gln Ser Ala Gly Ile Ala Thr Thr Ile Val Glu Ala
15     20     25     30
Arg Asp Lys Pro Gly Gly Arg Ala Tyr Val Trp Asn Asp Gln Gly His
35     40     45
Val Phe Asp Ala Gly Pro Thr Val Val Thr Asp Pro Asp Ser Leu Arg
20     50     55     60
Glu Leu Trp Ala Leu Ser Gly Gln Pro Met Glu Arg Asp Val Thr Leu
65     70     75     80
Leu Pro Val Ser Pro Phe Tyr Arg Leu Thr Trp Ala Asp Gly Arg Ser
85     90     95
Phe Glu Tyr Val Asn Asp Asp Asp Glu Leu Ile Arg Gln Val Ala Ser
100    105    110
Phe Asn Pro Ala Asp Val Asp Gly Tyr Arg Arg Phe His Asp Tyr Ala
115    120    125
Glu Glu Val Tyr Arg Glu Gly Tyr Leu Lys Leu Gly Thr Thr Pro Phe
130    135    140
Leu Lys Leu Gly Gln Met Leu Asn Ala Ala Pro Ala Leu Met Arg Leu
145    150    155    160
Gln Ala Tyr Arg Ser Val His Ser Met Val Ala Arg Phe Ile Gln Asp
165    170    175
Pro His Leu Arg Gln Ala Phe Ser Phe His Thr Leu Leu Val Gly Gly
180    185    190
Asn Pro Phe Ser Thr Ser Ser Ile Tyr Ala Leu Ile His Ala Leu Glu
195    200    205
Arg Arg Gly Gly Val Trp Phe Ala Lys Gly Gly Thr Asn Gln Leu Val
210    215    220
Ala Gly Met Val Ala Leu Phe Thr Arg Leu Tyr Tyr Thr Leu Leu Leu
225    230    235    240
Asn Ala Arg Val Thr Arg Ile Asp Thr Glu Gly Asp Arg Ala Thr Gly
245    250    255
Val Thr Leu Leu Asp Gly Arg Gln Leu Arg Ala Asp Thr Val Ala Ser
260    265    270
Asn Gly Asp Val Met His Ser Tyr Arg Asp Leu Leu Gly His Thr Arg
275    280    285
Arg Gly Arg Thr Lys Ala Ala Ile Leu Asn Arg Gln Arg Trp Ser Met
290    295    300

```

# EP 0 872 554 A2

5 Ser Leu Phe Val Leu His Phe Gly Leu Ser Lys Arg Pro Glu Asn Leu  
 305 310 315 320  
 Ala His His Ser Val Ile Phe Gly Pro Arg Tyr Lys Gly Leu Val Asn  
 325 330 335  
 Glu Ile Phe Asn Gly Pro Arg Leu Pro Asp Asp Phe Ser Met Tyr Leu  
 340 345 350  
 10 His Ser Pro Cys Val Thr Asp Pro Ser Leu Ala Pro Glu Gly Met Ser  
 355 360 365  
 Thr His Tyr Val Leu Ala Pro Val Pro His Leu Gly Arg Ala Asp Val  
 370 375 380  
 Asp Trp Glu Ala Glu Ala Pro Gly Tyr Ala Glu Arg Ile Phe Glu Glu  
 385 390 395 400  
 15 Leu Glu Arg Arg Ala Ile Pro Asp Leu Arg Lys His Leu Thr Val Ser  
 405 410 415  
 Arg Ile Phe Ser Pro Ala Asp Phe Ser Thr Glu Leu Ser Ala His His  
 420 425 430  
 20 Gly Ser Ala Phe Ser Val Glu Pro Ile Leu Thr Gln Ser Ala Trp Phe  
 435 440 445  
 Arg Pro His Asn Arg Asp Arg Ala Ile Pro Asn Phe Tyr Ile Val Gly  
 450 455 460  
 Ala Gly Thr His Pro Gly Ala Gly Ile Pro Gly Val Val Gly Ser Ala  
 465 470 475 480  
 25 Lys Ala Thr Ala Gln Val Met Leu Ser Asp Leu Ala Val Ala  
 485 490

## (2) INFORMATION FOR SEQ ID NO: 12:

30 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 1482 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

40 ATGAGTTCCG CCATCGTCAT CGGCGCAGGT TTCGGCGGGC TTGCGCTTGC CATCCGCTG 60  
 CAATCGGCCG GCATCGCGAC CACCATCGTC GAGGCCCGCG ACAAGCCCGG CGGCCGCGCC 120  
 TATGTCTGGA ACGATCAGGG CCACGTCTTC GATGCAGGCC CGACGGTCGT GACCGACCCC 180  
 GACAGCCTGC GAGAGCTGTG GGCCCTCAGC GGCCAACCGA TGGAGCGTGA CGTGACGCTG 240  
 CTGCCGGTCT CGCCCTTCTA CCGGCTGACA TGGGCGGACG GCCGCAGCTT CGAATACGTG 300  
 45 AACGACGACG ACGAGCTGAT CCGCCAGGTC GCCTCCTTCA ATCCCGCCGA TGTGATGGC 360  
 TATCGCCGCT TCCACGATTA CGCCGAGGAG GTCTATCGCG AGGGGTATCT GAAGCTGGGG 420  
 ACCACGCCCT TCCTGAAGCT GGGCCAGATG CTGAACGCCG CGCCGGCGCT GATGCGCCTG 480  
 CAGGCATACC GCTCGGTCCA CAGCATGGTG GCGCGCTTCA TCCAGGACCC GCATCTGCGG 540  
 50 CAGGCCTTCT CGTCCACAC GCTGCTGGTC GCGGGGAACC CGTTTTCGAC CAGCTCGATC 600  
 TATGCGCTGA TCCATGCGCT GGAACGGCGC GCGGCGTCT GGTTCGCCAA GGGCGGCACC 660

55

# EP 0 872 554 A2

```

AACCAGCTGG TCGCGGGCAT GGTCGCCCTG TTCGAGCGTC TTGGCGGCAC GCTGCTGCTG      720
AATGCCCGCG TCACGCGGAT CGACACCGAG GGCATCGCG CCACGGGCGT CACGCTGCTG      780
5  SAGCGGCGGC AGTTGCGCGC GGATACGGTG GCCAGCAACG GCGACGTGAT GCACAGCTAT      840
CGCGACCTGC TGGGCCATAC CCGCCGCGGG CGCACCAAGG CCGCGATCCT GAACCGGCAG      900
CGCTGGTCGA TGTGCTGTT CGTGCTGCAT TTCGGCCTGT CCAAGCGCCC CGAGAACCTG      960
10  GCCCACCACA GCGTCATCTT CGGCCGCGC TACAAGGGGC TGGTGAACGA GATCTTCAAC     1020
GGGCCACGCC TGGCGGACGA TTCTCGATG TATCTGCATT CGCCCTGCGT GACCGATCCC     1080
AGCCTGGCCC CCGAGGGGAT GTCCACGCAT TACGTCTTG CGCCCGTTCC GCATCTGGGC     1140
CGCGCGGATG TCGATGCGA AGCGGAGGCT CCGGGCTATG CCGAGCGCAT CTTGAGGAA     1200
15  CTGGAGCGCC GCGCCATCCC CGACCTGCGC AAGCACCTGA CCGTCAGCCG CATCTTCAGC     1260
CCCGCCGATT TCAGCACCGA ACTGTCGGCC CATCACGGCA GCGCCTTCTC GGTGAGCCG     1320
ATCTTGACGC AATCCGCCCTG GTCCGCCCC CATAACCGCG ACCGCGCGAT CCCGAAC TTC     1380
20  TATATCTGG GGGCGGGCAC GCATCCGGGT GCGGGCATCC CGGGTGTCGT TGGCAGCGCC     1440
AAGGCCACGG CGCAGGTCAT GCTGTGGGAC CTGGCCGTCG CA                        1482

```

12 INFORMATION FOR SEQ ID NO: 13:

```

25  (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 382 amino acids
      (B) TYPE: amino acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

```

(ii) MOLECULE TYPE: protein

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

```

Met Ser His Asp Leu Ile Ala Gly Ala Gly Leu Ser Gly Ala Leu
1      5      10      15
35  Ile Ala Leu Ala Val Arg Asp Arg Arg Pro Asp Ala Arg Ile Val Met
      20      25      30
Leu Asp Ala Arg Ser Gly Pro Ser Asp Gln His Thr Trp Ser Cys His
      35      40      45
40  Asp Thr Asp Leu Ser Pro Glu Trp Leu Ala Arg Leu Ser Pro Ile Arg
      50      55      60
Arg Gly Glu Trp Thr Asp Gln Glu Val Ala Phe Pro Asp His Ser Arg
      65      70      75      80
Arg Leu Thr Thr Gly Tyr Gly Ser Ile Glu Ala Gly Ala Leu Ile Gly
      85      90      95
45  Leu Leu Gln Gly Val Asp Leu Arg Trp Asn Thr His Val Ala Thr Leu
      100      105      110
Asp Asp Thr Gly Ala Thr Leu Thr Asp Gly Ser Arg Ile Glu Ala Ala
      115      120      125
50  Cys Val Ile Asp Ala Arg Gly Ala Val Glu Thr Pro His Leu Thr Val
      130      135      140
Gly Phe Gln Lys Phe Val Gly Val Glu Ile Glu Thr Asp Ala Pro His

```

55

# EP 0 872 554 A2

145 150 155 160  
 Gly Val Glu Arg Pro Met Ile Met Asp Ala Thr Val Pro Gln Met Asp  
 165 170 175  
 Gly Tyr Arg Phe Ile Tyr Leu Leu Pro Phe Ser Pro Thr Arg Ile Leu  
 180 185 190  
 Ile Glu Asp Thr Arg Tyr Ser Asp Gly Gly Asp Leu Asp Asp Gly Ala  
 195 200 205  
 Leu Ala Gln Ala Ser Leu Asp Tyr Ala Ala Arg Arg Gly Trp Thr Gly  
 210 215 220  
 Gln Glu Met Arg Arg Glu Arg Gly Ile Leu Pro Ile Ala Leu Ala His  
 225 230 235 240  
 Asp Ala Ile Gly Phe Trp Arg Asp His Ala Gln Gly Ala Val Pro Val  
 245 250 255  
 Gly Leu Gly Ala Gly Leu Phe His Pro Val Thr Gly Tyr Ser Leu Pro  
 260 265 270  
 Tyr Ala Ala Gln Val Ala Asp Ala Ile Ala Ala Arg Asp Leu Thr Thr  
 275 280 285  
 Ala Ser Ala Arg Arg Ala Val Arg Gly Trp Ala Ile Asp Arg Ala Asp  
 290 295 300  
 Arg Asp Arg Phe Leu Arg Leu Leu Asn Arg Met Leu Phe Arg Gly Cys  
 305 310 315 320  
 Pro Pro Asp Arg Arg Tyr Arg Leu Leu Gln Arg Phe Tyr Arg Leu Pro  
 325 330 335  
 Gln Pro Leu Ile Glu Arg Phe Tyr Ala Gly Arg Leu Thr Leu Ala Asp  
 340 345 350  
 Arg Leu Arg Ile Val Thr Gly Arg Pro Pro Ile Pro Leu Ser Gln Ala  
 355 360 365  
 Val Arg Cys Leu Pro Glu Arg Pro Leu Leu Gln Glu Arg Ala  
 370 375 380

## (2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 1149 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

ATGAGCCATG ATCTGCTGAT CGCGGGCGCG GGGCTGTCCG GTGCGCTGAT CGCGCTTGCC 60  
 GTTCGCGACC GCAGACCGGA TCGCGGCATC GTGATGCTCG ACGCGCGGTC CGGCCCCCTCG 120  
 GACCAGCACA CCTGGTCCTG CCACGACACG GATCTTTTCG CCGAATGCTG GCGCGCGCTG 180  
 TCGCCCATTC GTCGCGGCGA ATGGACGGAT CAGGAGGTCG CGTTTCCCGA CCATTCGCGC 240  
 CGCCTGACGA CAGGCTATGG CTCGATCGAG GCGGGCGCGC TGATCGGGCT GCTGCAGGGT 300  
 GTCGATCTGC GGTGGAATAC GCATGTCGCG ACGCTGGACG ATACCGGCGC GACGCTGACG 360  
 GACGGCTCGC GGATCGAGGC TGCCTGCGTG ATCGACGCCC GTGGTGCCGT CGAGACCCCG 420

# EP 0 872 554 A2

CACCTGACCG TGGGTTTCCA GAAATTCGTG GGCCTCGAGA TCGAGACCGA CGCCCCCAT 480  
 GGCCTCGAGC GCCCAGATGAT CATGGACGCG ACCGTTCCGC AGATGGACGG GTACCGCTTC 540  
 5 ATCTATCTGC TGCCCTTCAG TCCCACCCGC ATCCTGATCG AGGATACGCG CTACAGCGAC 600  
 GCGGCGGATC TGGACGATGG CGCGCTGGCG CAGGCGTCGC TGGACTATGC CGCCAGGCGG 660  
 GGCTGGACCG GGCAGGAGAT GCGGCGCGAA AGGGGCATCC TGCCCATCGC GCTGGCCCAT 720  
 10 GACGCCATAG GCTTCTGGCG CGACCACGCG CAGGGGGCGG TGCCGGTTGG GCTGGGGGCA 780  
 GGGCTGTTCC ACCCCGTCAC CGGATATTTC CTGCCCTATG CCGCGCAGGT CGCGGATGCC 840  
 ATCGCGGCGC GCGACCTGAC GACCGCGTCC GCCCGTCGCG CGGTGCGCGG CTGGGCCATC 900  
 GATCGCGCGG ATCGCGACCG CTTCTGCGG CTGCTGAACC GGATGCTGTT CCGCGGCTGC 960  
 15 CCGCCCGACC GTCGCTATCG CCTGCTGCAG CGGTTCTACC GCCTGCCGCA GCCGCTGATC 1020  
 GAGCGCTTCT ATGCCGGGCG CCTGACATTG GCCGACCGGC TTCGCATCGT CACCGGACGC 1080  
 CCGCCCATTC CGCTGTCGCA GGCCGTGCGC TGCCTGCCCG AACGCCCCCT GCTGCAGGAG 1140  
 20 AGAGCATGA 1149

## (2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 169 amino acids  
 (B) TYPE: amino acid  
 25 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear  
  
 (ii) MOLECULE TYPE: protein  
  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:  
 Met Ser Thr Trp Ala Ala Ile Leu Thr Val Ile Leu Thr Val Ala Ala  
 1 5 10 15  
 Met Glu Leu Thr Ala Tyr Ser Val His Arg Trp Ile Met His Gly Pro  
 20 25 30  
 35 Leu Gly Trp Gly Trp His Lys Ser His His Asp Glu Asp His Asp His  
 35 40 45  
 Ala Leu Glu Lys Asn Asp Leu Tyr Gly Val Ile Phe Ala Val Ile Ser  
 50 55 60  
 40 Ile Val Leu Phe Ala Ile Gly Ala Met Gly Ser Asp Leu Ala Trp Trp  
 65 70 75 80  
 Leu Ala Val Gly Val Thr Cys Tyr Gly Leu Ile Tyr Tyr Phe Leu His  
 85 90 95  
 Asp Gly Leu Val His Gly Arg Trp Pro Phe Arg Tyr Val Pro Lys Arg  
 100 105 110  
 45 Gly Tyr Leu Arg Arg Val Tyr Gln Ala His Arg Met His His Ala Val  
 115 120 125  
 His Gly Arg Glu Asn Cys Val Ser Phe Gly Phe Ile Trp Ala Pro Ser  
 130 135 140  
 50 Val Asp Ser Leu Lys Ala Glu Leu Lys Arg Ser Gly Ala Leu Leu Lys  
 145 150 155 160

55

# EP 0 872 554 A2

Asp Arg Glu Gly Ala Asp Arg Asn Thr  
165

## (2) INFORMATION FOR SEQ ID NO: 16:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 506 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

ATGAGCACTT	GGGCCGCAAT	CCTGACCGTC	ATCCTGACCG	TCGCCGCGAT	GGAGCTGACG	60
GCCTACTCCG	TCCATCGGTG	GATCATGCAT	GGCCCCCTGG	GCTGGGGCTG	GCATAAATCG	120
CACCAAGCAG	AGGATCACGA	CCACGCGCTC	GAGAAGAACG	ACCTCTATGG	CGTCATCTTC	180
CGGCTAATCT	CGATCGTGCT	GTCGCGATC	GGCGCGATGG	GGTCGGATCT	GGCCTGGTGG	240
CTGGCGGTGG	GGGTCACCTG	CTACGGGGCTG	ATCTACTATT	TCCTGCATGA	CGGCTTGGTG	300
CATGGGCGCT	GGCCGTTCGG	CTATGTCCCC	AAGCGCGGCT	ATCTTCGTCG	CGTCTACCAG	360
GCACACAGGA	TGCATCACGC	GGTCCATGGC	CGCGAGAACT	GCCTCAGCTT	CGGTTTCATC	420
TGGGCGGCTT	CGGTCGACAG	CCTCAAGGCA	GAGCTGAAAC	GCTCGGGCGC	GCTGCTGAAG	480
GACCGGCAAG	GGCGGGATCG	CAATAC				506

## (2) INFORMATION FOR SEQ ID NO: 17:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 726 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

ATGTCCGGTC	GTAAACCGGG	TACCACCGGT	GACACCATCG	TTAACCTGGG	TCTGACCGCT	60
GCTATCCTGC	TGTGCTGGCT	GGTTCACAC	GCTTTCACCC	TGTGGCTGCT	GGACGCTGCT	120
GCTCACCCGC	TGCTGGCTGT	TCTGTGCCTG	GCTGGTCTGA	CCTGGCTGTC	CGTTGGTCTG	180
TTCATCATCG	CTCACGACGC	TATGCACGGT	TCCGTTGTTC	CGGGTCGTCC	GCGGGCTAAC	240
GCTGCTATCG	GTCAGCTGGC	TCTGTGGCTG	TACGCTGGTT	TCTCCTGGCC	GAAACTGATC	300
GCTAAACACA	TGACCCACCA	CCGTCACGCT	GGTACCGACA	ACGACCCGGA	CTTCGGTCAC	360
GGTGGTCCGG	TTCGTTGGTA	CGGTTCCCTC	GTTTCCACCT	ACTTCGGTTG	GCGTGAAGGT	420
CTGCTGCTGC	CGGTTATCGT	TACCACCTAC	GCTCTGATCC	TGGGTGACCG	TTGGATGTAC	480
GTTATCTTCT	GGCCGGTTCC	GGCTGTTCTG	GCTTCCATCC	AGATCTTCGT	TTTCGGTACC	540
TGGCTGCCGC	ACCGTCCGGG	TCACGACGAC	TTCCCGGACC	GTCACAACGC	TCGTTCCACC	600
GGTATCGGTG	ACCCGCTGTC	CCTGCTGACC	TGCTTCCACT	TCGGTGGTTA	CCACCACGAA	660

CACCACCTGC ACCCGCACGT TCCGTGGTGG CGTCTGCCGC GTACCCGTAA AACCGGTGGT 720  
 5 CGTGCT 726

## Claims

1. A process for the preparation of canthaxanthin by culturing under suitable culture conditions a cell which is transformed by a DNA sequence comprising the following DNA sequences:

a) a DNA sequence which encodes the GGPP synthase of *Flavobacterium* sp. R1534 (crtE) or a DNA sequence which is substantially homologous;

b) a DNA sequence which encodes the prephytoene synthase of *Flavobacterium* sp. R1534 (crtB) or a DNA sequence which is substantially homologous;

c) a DNA sequence which encodes the phytoene desaturase of *Flavobacterium* sp. R1534 (crtI) or a DNA sequence which is substantially homologous;

d) a DNA sequence which encodes the lycopene cyclase of *Flavobacterium* sp. R1534 (crtY) or a DNA sequence which is substantially homologous;

e) a DNA sequence which encodes the  $\beta$ -carotene  $\beta$ 4-oxygenase of the microorganism E-396 (FERM BP-4283) [crtW<sub>E396</sub>] or a DNA sequence which is substantially homologous;

or a cell which is transformed by a vector comprising DNA sequences specified above under a) to e) and by isolating canthaxanthin from such cells or the culture medium by methods known in the art.

2. A process for the preparation of a mixture of adonixanthin and astaxanthin or adonixanthin or astaxanthin alone by a process as claimed in claim 1 characterized therein that in addition to the DNA sequences specified in claim 1 under a) to e) the following additional DNA sequence is present:

f) a DNA sequence which encodes the  $\beta$ -carotene hydroxylase of the microorganism E-396 (FERM BP-4283) [crtZ<sub>E396</sub>] or a DNA sequence which is substantially homologous;

and the DNA sequence specified under e) of claim 1 is as specified in claim 1 or the following sequence:

g) a DNA sequence which encodes the  $\beta$ -carotene  $\beta$ 4-oxygenase of *Alcaligenes* strain PC-1 (crtW) or a DNA sequence which is substantially homologous;

and isolating the desired mixture of adonixanthin and astaxanthin or adonixanthin or a astaxanthin alone from such cells of the culture medium and separating the desired mixture or carotenoids alone from other carotenoids which might be present by methods known in the art.

3. A process for the preparation of zeaxanthin by a process as claimed in claim 1 characterized therein that the DNA sequence as specified under e) is replaced by the DNA sequence as specified under f) in claim 2 and by isolating zeaxanthin from the cell or the culture medium and separating it from other carotenoids which might be present by methods known in the art.

4. A process for the production of adonixanthin by culturing under suitable culture conditions a cell which is transformed by a DNA sequence comprising the following heterologous DNA sequences:

a) a DNA sequence which encodes the GGPP synthase of the microorganism E-396 (FERM BP-4283)

[crtE<sub>E396</sub>] or a DNA sequence which is substantially homologous;

b) a DNA sequence which encodes the prephytoene synthase of the microorganism E-396 (FERM BP-4283) [crtB<sub>E396</sub>] or a DNA sequence which is substantially homologous;

c) a DNA sequence which encodes the phytoene desaturase of the microorganism E-396 (FERM BP-4283) [crtI<sub>E396</sub>] or a DNA sequence which is substantially homologous;

d) a DNA sequence which encodes the lycopene cyclase of the microorganism E-396 (FERM BP-4283) [crtY<sub>E396</sub>] or a DNA sequence which is substantially homologous;

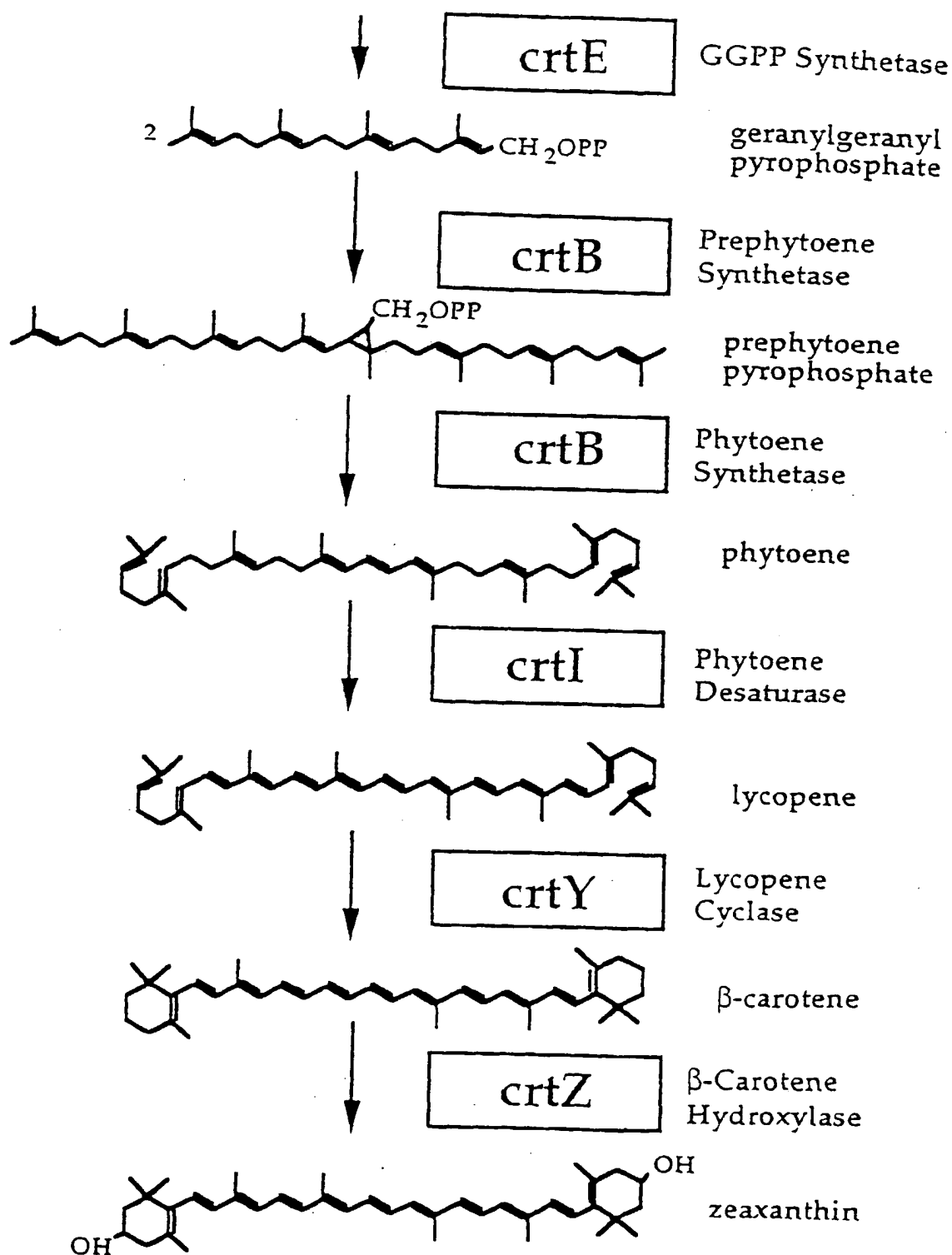
e) a DNA sequence which encodes the  $\beta$ -carotene hydroxylase of the microorganism E396 (FERM BP-4283) [crtZ<sub>E396</sub>] or a DNA sequence which is substantially homologous; and

f) a DNA sequence which encodes the  $\beta$ -carotene  $\beta$ 4-oxygenase of the microorganism E396 (FERM BP-4283) [crtW<sub>E396</sub>] or a DNA sequence which is substantially homologous;

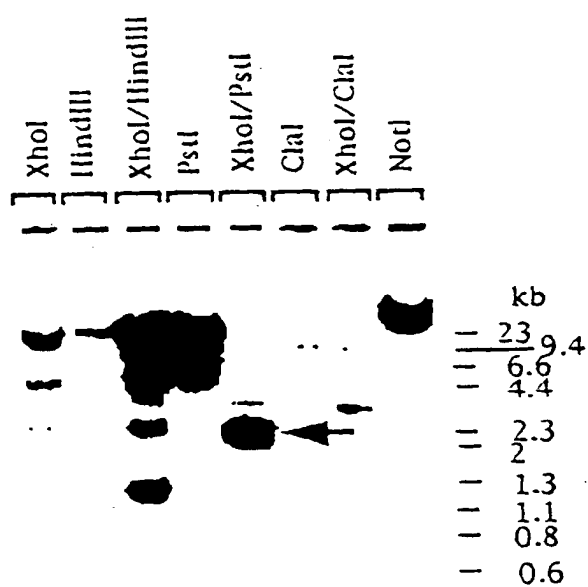
and isolating adonixanthin from the cell or the culture medium and separating it from other carotenoids which might be present by methods known in the art.

5. A process for the preparation of a food or feed composition characterized therein that after a process as claimed in any one of claims 1 to 4 has been effected the carotenoid or carotenoid mixture is added to food or feed.
6. A process as claimed in any one of claims 1 to 5 characterized therein that the transformed host cell is a prokaryotic host cell, like E. coli, Bacillus or Flavobacter.
7. A process as claimed in any one of claims 1 to 5 characterized therein that the transformed host cell is a eukaryotic host cell, like yeast or a fungal cell.

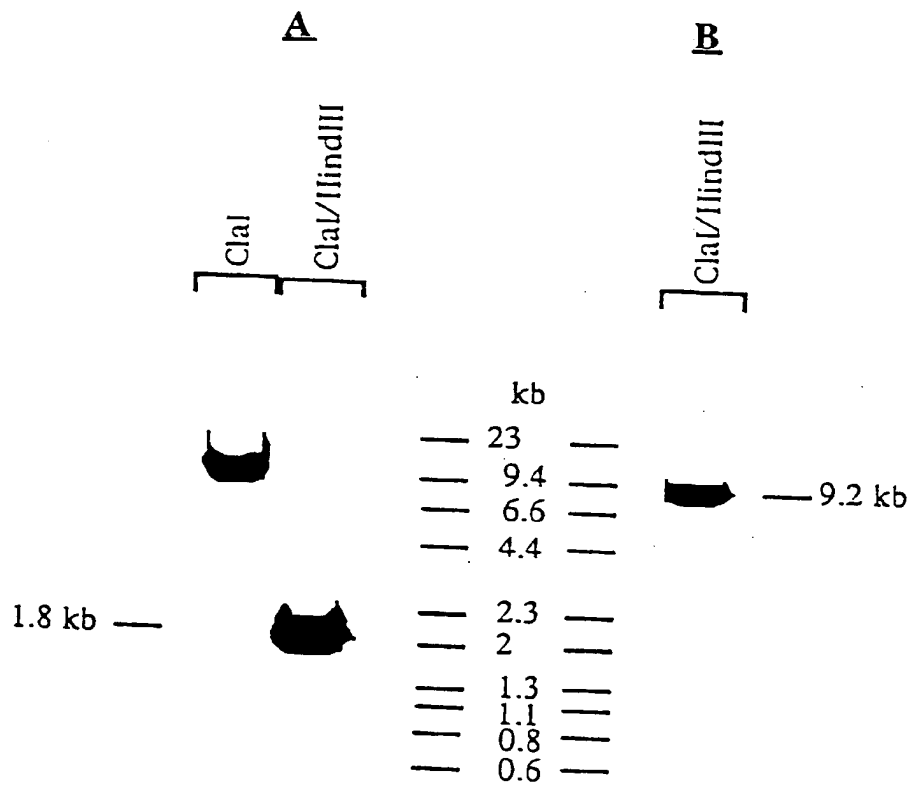
Fig. 1



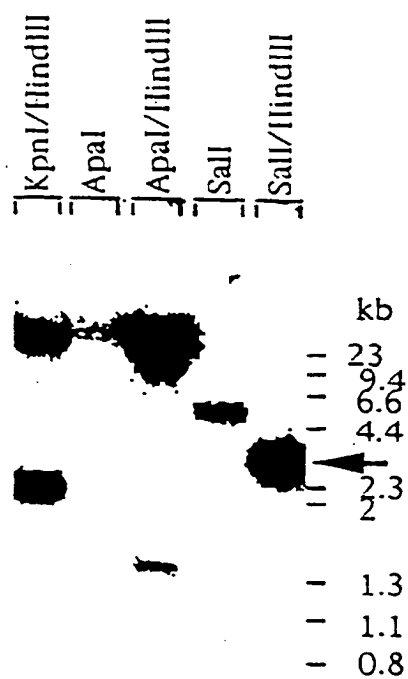
**Fig. 2**



**Fig. 3**



**Fig. 4**



**Fig. 5**

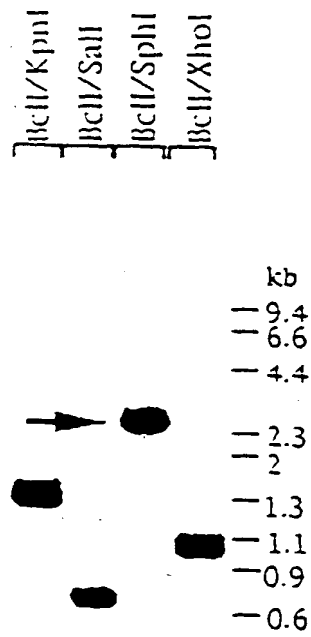


Fig. 6

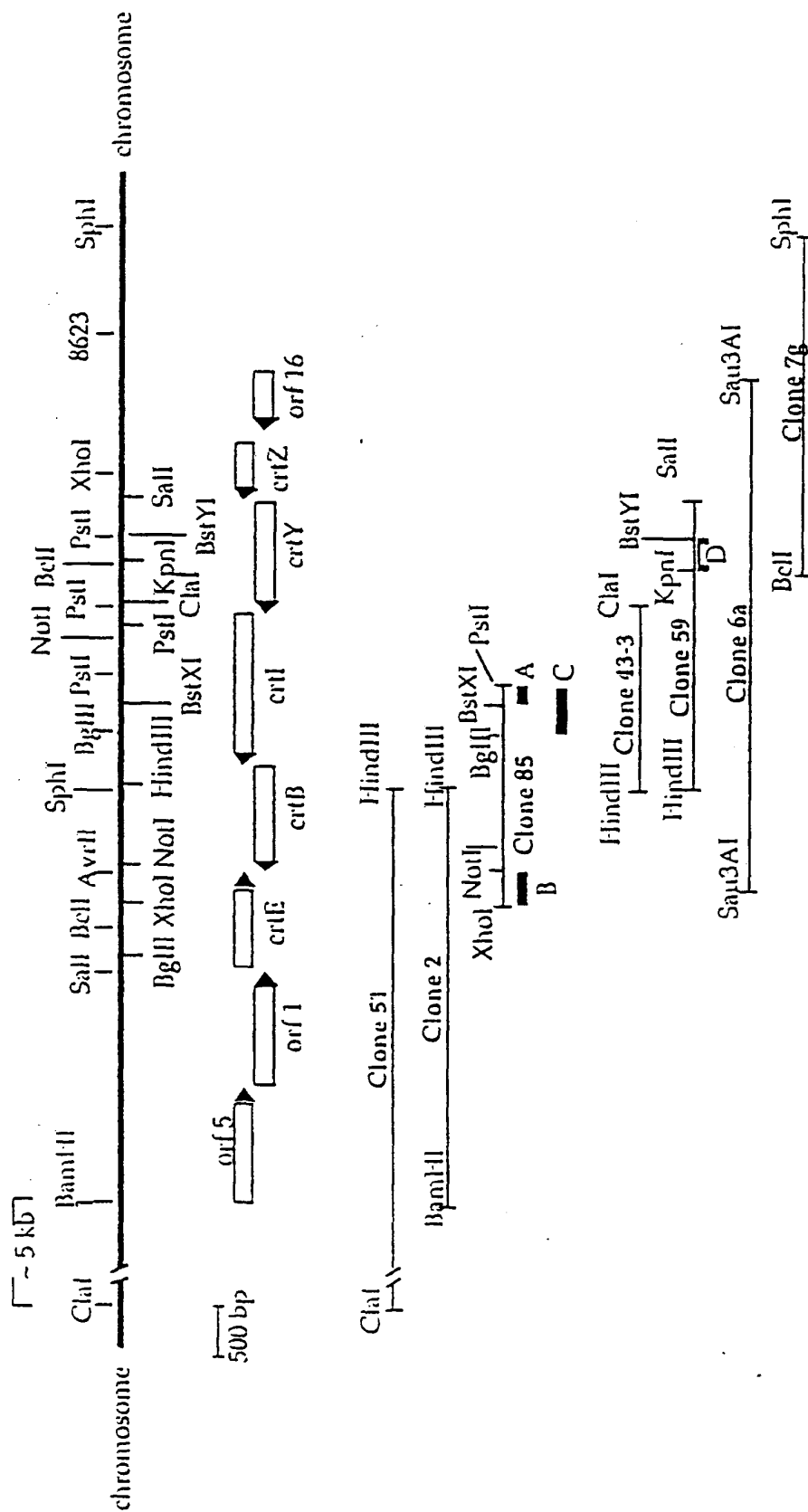


Fig. 7/1

1	GGATCCGGCGCTGGCGCTTCGGATCAGACGGCGCTTGGCGATCGGTC	301	AGATCATGTCTGATTCATGCCCCGTCTATTGCAAAACCGATCAACGATCC	350
	CCTAGCGCGGACCGCGAAGCGCTAGTGTGTCGGCGGAAACGCTAGCCAG		TCTACTACACGCTAGCTACCGCAGTAACGCTTTTGGCTAGTGGCTAGG	
orf-5 -->	D P R L A V R D Q Q P P L R I G Q		D D V L I M G P S L Q N R S P I L	
51	AGCATATCCCATGAAACCGACGGGACGACGAGCGGCGGCGCGGATC	351	TGTCGGGTGATGGCATTTGTTTGCATGCCCGGAGGGCTAGATGGGCGA	400
	TGCTATAGGGGTACTTGGCGTCCGCTGCTGCTGCGCGCGGCGGCTCTAG		ACAGCGCACTACGTAACAAACGTTACGGGGCTCCGATCCTACCGCGCT	
	H H P H E P Q R T T Q R A P Q I		S R D G I V C N A P R A R M A R	
101	GGGCGGCTCCAGCACGGCATGCGCCATCATGCGGAGGCGCGCGGCGCA	401	AGCATCAAGGGGGGAGAGACATGMAATCGAGGAGCGGCTCTTTGTGCT	450
	CCCGCGCAGGTCGTGCGGTACGGGTAGTAGCGCTTCGGGGGCGCGCGT		TCCTAGTTCGCCCTCTCTCTGTACTTTAGCTCCTGCCCAAGAACAGCA	
	G R V Q H G M R H R E G P R R H		R I K G R D M E I E G R V F V V	
151	TGGGGGGGTGGCCATTCGCAAGAACTCGCAGGCTGTCCGCTGCGCAAGG	451	CACGGCGCGCGCATTCGGGTCTGGGGGGCGCTTCGGCGCGGATGCTGGCCC	500
	ACCCGCGCACGGGTAAAGGCTTCTTGAGCGCTCGACAGGGACACGGTTCC		GTGCGCGCGCGCTAGCCCAAGACCCCGCGCGAGCGCGCGCTACGACCGGG	
	C A R A H S E E L A A C P L R K V		T G A A S G L G A A S A R M L A Q	
201	TCCGCGCAGATCGCGCGTATTCGATGCAATGACGGCGCGCATCGCGT	501	AAGCGCGCGGAAAGTCTGCTGGCGCGATCTGGCGGAACGAGACGCG	550
	ACCGCGGTAGCGCGCATAGGCTAGCTACGTGCGCGCGCTACGGCA		TTCCGCGCGCTTCAGACAGACCGGCTAGACCGCTTGGCTTCTGCGC	
	A P D R A V F R C S D G P D A R		G G A K V V L A D L A E P K D A	
251	CGGCGCGCGCTGCGCGCGGACGAGCGCATTCGCGACGAAACGCTTCGG	551	CCCGAAGCGCGGTTCAAGCGCGCTTCGAGCTGACGAGCGGACCGCGCTCC	600
	CCGCGCGGACGCGCGCGGCTGCTGCGGTAGCGCGCTTGGGAAGCG		GGGCTTCGCGCGCAAGTGGCGCGGACGCTGCACTGGCTGGCTGGCGACG	
	G P A L P R R H Q R I A H E P F R		P E G A V H A A C D V T D A T A A	

Fig. 7/2

601	GCACAGCGCCATCGCCCTGCGGACCGACCCGCTTCGGCAGGCTGGACGGCC	650	901	CGTGGCGGCATCAGCGCTGCGGATGCGCCCGCGACGCTTGCCCGGCACGGCA	950
	CGTCTCCCGCTAGCGGACCGCTGCTGGCGACCGCTGCGACCTGCGCGG			GCAACCGCGCTACTGCGACCGCTACCGGGCGCTGGAGCGCGCGCTGCGCGT	
	Q T A I A L A T D R F G R L D G L			V A G M T L P M A R D L A R H G I	
651	TTGTGAATGCGCGGCATCGCGCCGCGGACCGATGCTGGCGCGCGAC	700	951	TCCGCTCATGACCATGCGCCCGCGCATCTTCGCAACCCCGATGCTGGAG	1000
	AACACTTCAACCGCGCGTACGCGCGCGCGCTTGCGCTACGACCCCGCGCTG			AGCGGCACTACTGCTAGCGCGCGCGCTAGAGCGCGTGGCGCTACGACCTC	
	V N C A G I A P A E R M L G R D			R V M T I A P G I F R T P M L E	
701	GGCGCGCATGACTGGAACGCTTTGCGCGTGGCGTCAAGTCAACCTGAT	750	1001	GGCTGCGCGCAGGACGCTTCAGGACAGCTGGCGCGCGCGTGGCGCTTCCC	1050
	CCCGCGTACCTGACCTGTGGAACCGGACCGCAGCTGCTAGTTGGACTA			CCGACGGCGTCTGCGACGCTGTGCGACCGCGCGCGCGCGCGGAGGG	
	G P H G L D S F A R A V T I N L I			G L P Q D V Q D S L G A A V P F P	
751	CGGACGCTTCAACATGGCGCGCTTCAGCGGAGCGGATGGCGCGGACG	800	1051	CTCGCGGCTGGAGAGCGCGTGGGAATACCGGGCGCTTGCACCAATCA	1100
	GCGTGGAGTTGTACCGCGCGGAGCGTGGCTGCGCTACCGGGCGCTTGC			GAGCGCGCAGCCCTCTCGGCGAGCGCTTATGCGCGCGCGACACGCTGCTAGT	
	G S F N M A R L A A E A M A R N E			S R L G E P S E Y A A L L H H I I	
801	AGCCGCTCCGGCGGAGCGTGGCTGATGCTCAACAGCGCTCGATCGCG	850	1101	TGCGGACCCCATGCTGAACGAGAGGTCATCCCGCTCGACGGCGCATTG	1150
	TCGGGCGGCGCGCTGCGACCGCACTAGCAGTTGTGCGCGAGCTAGCGC			AGCGCTTGGGGTACGACTTGGCTCTCCAGTAGCGGAGCTGCGCGTAAC	
	P V R G E R G V I V N T A S I A			A N P M L N G E V I R L D G A L	
851	GGGACGACCGACACATCGGACAGGTGCGCTATGCGCGCGGAGGAGCGGG	900	1151	CGCATGGCGCGCGAGTGAAGGAGCGCTTCAATGGAACCGCATGCTCATCAC	1200
	CGGCTGCTGCTGTCTACCTGTCCAGCGGATACGGCGCGCTGCTTCCGCG			CGGTACCGCGCGTTCACCTTCTCCGCAAGTACCTGGGGTAGCAGTAGTGG	
	A Q D G Q I G Q V A Y A A S K A O			R M A P K A M D P I V I T	
				orf-1 -->	

Fig. 7/3

1201	G G C C C G A T G C C A C C C C G A T G G G G C A T T C C A G G G G A T C T T C C C G G A T -----+-----+-----+-----+-----+-----+-----+-----+-----+ C C G G C T A C C G C T G G G C T A C C C C G T A G G T C C C C T A G A A C G G C C T A G A M R T P M G A F Q G D L A A N	1250	1501	G T C G T C G C G G G G A T G G A G A G C A T G T C G A A C G C C C T A C C T C T C C T G C C -----+-----+-----+-----+-----+-----+-----+-----+-----+ C A G C A C G C C C C C T A C C T C T C T A C A G C T T C C G G G G A T G G A C A C G G V V A G G M E S M S H A P Y L L P	1550
1301	G G A T C C C C A C C C T T G C C G C G A C C G A T C C G C G C C G C T G A A C G C C C -----+-----+-----+-----+-----+-----+-----+-----+-----+ C C T A C G G C C T G G A A C C C C C C T G C C C T A G C C C G C G C G A C T T G C C G D A P T L G A D A I R A A L N G L	1300	1551	C A A G C G C G G T C G G G A T G C G C A T G C G C A T C A C C G T G T C T G C A T C A C A -----+-----+-----+-----+-----+-----+-----+-----+-----+ G T T C C C C C A C C C C T A C C G T A C C C G T A C T G C C A C A C C A C C T A G T G T K A R S G M R M G H D R V L D H M	1600
1351	T G T C C C C G A C A T G T G A C G A G G T C C T G A T G C C T G C G T C T C C C C G G -----+-----+-----+-----+-----+-----+-----+-----+-----+ A C A G G C C T G T A C C A C C T C C T C C A C G A C T A C T G T T C C C C G C G A C T A C C G S P D M V D E V L M G C V L A A	1350	1601	T G T T C T C G A C C G G T T G C A C A C C C C T A T G A A G G C C C C C T G A T G G G C -----+-----+-----+-----+-----+-----+-----+-----+-----+ A C A A G A G C T G C C C A C C C C T C C T C C G A T A C T G T T C C C C G C G A C T A C C G F L D G L E D A Y D K G R L M G	1650
1391	G C C A G G T C A C C A C C G C A C G T C A G C G C G C C T T G C C C C C G A C T C C C -----+-----+-----+-----+-----+-----+-----+-----+-----+ C C G C T C C C A G T C C G T C C C C T G C A C T C C C C C G A A C C C C C C C T G A C G G C Q Q Q A P A R Q A A L G A G L P	1400	1651	A C C T T C C C G A G A T T C C C C C C G A T C A C C G T T T C A C C C C G A G C C C A -----+-----+-----+-----+-----+-----+-----+-----+-----+ T G G A G C C C T C C T A A C C G C C C C T A G T G C C A A G T G G C C C C T C C G C G T T F A E D C A G D H G F T R E A Q	1700
1401	C C T G T C G A C G C C A C C A C C A T C A A C G A G A T G T C C G A T C C G C C A T G A -----+-----+-----+-----+-----+-----+-----+-----+-----+ C G A C A C T C C C C C T G C T G T G T A G T T G C T C T A C A C G C C T A G C C G T A C T L S T G T T T I H E M C G S G M K	1450	1701	G G A C G A C T A T C C G C T C A C C A G C C T G G C C C C G C C A G C A C C C A T C C C C A -----+-----+-----+-----+-----+-----+-----+-----+-----+ C C T C C T A T A C C G A C T C C T C G A C C G C C G G G T C C T C C G G T A C C G G T D D Y A L T S L A R A Q D A I A S	1750
1451	A G C C G C G A T C T G G C C A T G A C C T G A T C C C C C G C G A T C G C G C G C A T C -----+-----+-----+-----+-----+-----+-----+-----+-----+ T C C G C C C T A C G A C C C G T A C T G A C T A C C G C C C C T A C C G C C C G T A G A A M L G H D L I A A G S A G I	1500	1751	C C G G T G C C T T C C C C C G A G A T C C C C C C C T G A C C C T C A C C G C A C C A A G -----+-----+-----+-----+-----+-----+-----+-----+-----+ C G C C A C G A A C G C G C C T C T A G C G G G G C A C T G C A G T C C G C T G C G T T C G A F A A E I A P V T V T A R K	1800

Fig. 7/4

1801	GTCCAGACCAAGCTCGATACCGACGAGATCCCGCCAGAGCCCGCCCGGA CAGCTCTGGTGGCAGCTATCGCTCTCTACCGGGCCGTTCCGGCCGGGCT V Q T T V D T D E M P G K A R P E	1850	2101	TACCACTTCTTGAAGTCAACGAGGCAATTGCGCGTCTGTCGCCATGATCGC ATCTCCACAAAGTCCACTTCTCTCTGTAAGCGGCAGCAGCGGTACTAGCG Y D L F E V N E A F A V V A M I A	2150
1851	GAAGATCCCCCATCTGAAGCCCGCTTCCGTGACCGTGGCAGGTCAAGG CTTCTAGGGGTAGACTTCGGGGGAGAGGCACTGCCACCGTGGCACTGCC K I P H L K P A F R D G G T V T A	1900	2151	GATGAGGAGCTTGGGCTGCCACACGATGCCAGCAATCAACGGCGGGG CTACTTCTCGAACCGGACGCTGTCTGCTAGCGTCTGTAGTTGCCGCCCG M K E L G L P H D A T N I N G G A	2200
1901	CGCGAAGACGCTCGATCTCGACGGGGGGCGCGCTGTGATGATG GCGCTTGTGAGCAGCTAGACGCTGCGCCCGCGCGGACCACTACTAC A N S S S I S D G A A A L V M M	1950	2201	CCTGCCGCTTGGGCATCCCATCGGCGGCTCGGGGGCGCGATCATGGTC GGACGGCGAACCCTAGGTAGCGCGGACGCCCGCGCGCTAGTACCAG C A L G H P I G A S G A R I M V	2250
1951	CGCCAGTCGACGGCGAGAGCTGGGCTGACCGCGATCGCGCGGATCAT CGGTCAGCGTCCGGCTCTTCGACCGGACTGGGCTAGCGGGGCTAGTA R Q S Q A E K L G L T P I A R I I	2000	2251	ACGCTGCTGAACGGGATGGCGGCGCGGGCGGACCGCGGGGGCGCGATC TCCGACGACTTGGCTACCGCGCGCGCGCGCGCTGGCGCGCGCGCGGTAG T L L N A M A A R G A T R G A A S	2300
2001	CGGTATCGGACCCATGCGGACCGTCCCGGCTGTTCGCGACGGCCCGCA GCCAGTACGCTGGGTACGGCTGCCAGGGCCGACAGGGGCTGCCGGGGT G H A T H A D R P G L F P T A P I	2050	2301	CGTCTGCATCGCGGGGGCGGAGCGGACGGCCATGCGCGCTGGAACGGCTGA GCAGAGTAGCGCGCCCGCGCTCCGCTGCGGCTAGCGCGACCTTGGCGACT V C I G G G E A T A I A L E R L S	2350
2051	TGGCGCGATCGGCAAGCTCTGACCGGACGGACACCGCGCTTGGCGAT AGCCCGCTACGCTTCGACGACCTGGCGTCCCTGTGGGGGAGACCGCTA G A M R K L L D R T D T R L G D	2100	2351	CCTAATTCATTGGCGAATCGCGCTTTTCTGTCACGATCGGGGAAACCG CGATTAGTAACCGGCTTAGCGCCAAAGCACACGCTGCTACCCCTTGGC *	2400

Fig. 7/5

2401	GAAGCGGCGACGCCCTGTGTGTGGTGGCTGCACTGCTCTTCGGGCGCATGCC -----+-----+-----+-----+-----+-----+-----+ CTTTGCCCGGTGGGACCAACACCAACCGCAGCTGGACAGAAACCGCGGTACGG	2450	2701	GTCTGCGATGCCGATGGTGGATGCCCGCTGGCGGTGGAGATGGTTCATGCG -----+-----+-----+-----+-----+-----+-----+ CAGACGCTACGCTACCAAGCTACGAGCTACGGCGGACCGCCCACTCTTACAGGTACG	2750
				V C D A M V D A A C A V E M V H A	
2451	CGTACGGCATGTGGAGGGGCAATGGCGGCTTGGCGATCGGCTGGCATGA -----+-----+-----+-----+-----+-----+-----+ GCACTGGCGTACACCGTCCGCTACCCGCAACGGCTAGGCCAGCGTACT	2500	2751	CGCATCGCTGATCTTCGACGCAATGCCCTGCAATGGACGATGCCAGACCC -----+-----+-----+-----+-----+-----+-----+ GGTAGCGACTAGAACTGCTCTGTACGGGACGTACCTGCTACGGTCTCTGGG	2800
				A S L I F D D M P C M D D A R T R	
2501	CTGACGCAACGAAGCAACCAATGACGCCCAAGCAGCAATTCGCCCTACCG -----+-----+-----+-----+-----+-----+-----+ CACTGGCTTCTTCCGTGGCTACTCGCGGTTCTGCTTAAGGGGCAATCG	2550	2801	GTCCCGGTACGCCCGGCCCAATGTGGCCATGGCCAGGGCGCGCGGTG -----+-----+-----+-----+-----+-----+-----+ CAGGCGCATGTCGGCGGTGGGTACAGCGGGTACCGCTCCCGCGCGCCAC	2850
	crTE --> M T P K Q Q F P L R			R G Q P A T H V A H G E G R A V	
2551	GATCTGTGAGATCAGGCTGGCGCAGATCTCGGGCGAGTTCCGGTGGT -----+-----+-----+-----+-----+-----+-----+ CTACACCACTCTAGTCCGACCGCGCTAGACCGCGGTCAAGCCGCAACA	2600	2851	CTTGGGGCATGCGCCCTGATCAGCGAGGCCATGGCGATTTTGGCGGAGGC -----+-----+-----+-----+-----+-----+-----+ GAAAGCCGTAAGCGGACTAGTGGCTCCGCTCCCGTAAACCGGCTCCG	2900
	D L V E I R L A Q I S G Q F G V V			L A G I A L I T E A M R I L G E A	
2601	CTCGGCGCGCTCGGCGCGCCATCAGCGATCGCGCGCTCTCCCGCGCA -----+-----+-----+-----+-----+-----+-----+ GAGCGGGCGAGCGCGCGCTACTCGCTACGGCGGCAACAGCGCGCGGT	2650	2901	CGCGCGCGCTCGCGCTAGTGGCGGTTCCGACCGGTAGGTACAGCG -----+-----+-----+-----+-----+-----+-----+ R G A T P D Q R A R L V A S M S R	2950
	S A P L G A A M S D A A L S P G K				
2651	AACCTTTCGCGCGTCTGATCTGATGGTCCCGCAACCTTCGGCGCGG -----+-----+-----+-----+-----+-----+-----+ TTCCGAAGCGCGGACGACTACGACTACCGAGCGGCTTTGAGCGCGCGC	2700	2951	CGCGATGGGACCGGTGGGCTGTGGCAAGGCGAGGATCTGCACTGCAC -----+-----+-----+-----+-----+-----+-----+ CGCGTACCTGGCGACCCGACAGCGCTCCGCTCTAGACCTGGACGTG	3000
	R F R A V L M L M V A E S S G G			A M G P V G L C A G Q D L D L H	

Fig. 7/6

3001	GCOCOCAGACGCGCGCGATCGAACGTGACACAGACCTCAAGACGG -----+-----+-----+-----+-----+ CGGGGTTCCTCGCGCGCGCTAGCTTCCACTGTCTCTGAGTCTCTGCGC	3050	3301	AOCOCGCGCAGCTGACAGCTGATGCGCACCGCGCTGTTCGCGCGGG -----+-----+-----+-----+-----+ TCGGCGCGCTTGCACCTCTGACTACGCGTGGCGCGACAGCGCGCGCGC	3350
	A P K D A A G I E R E Q D L K T G			S R A Q L D E L M R T R L F R G G	
3051	CCTCTGTTCGTCGCGCGCTCGAGATGCTGTCCATTTATAGGCTCTGG -----+-----+-----+-----+-----+ GCACGACAGCGCGCGCGAGCTCTACGACAGCTAATTAATTCACAGCC	3100	3351	GCAGATCGCGGACCTCTGCGCGCGCTGCTGCGCGCATGACATCGCGCGCA -----+-----+-----+-----+-----+ CGCTAGCGGCTGACGACGCGCGCGCGCACGACGGCTACTGTAGGGCGGT	3400
	V L F V A G L E M L S I I K G L D			Q I A D L L A R V L P H D I R R S	
3101	ACAGCGCGAGACCGAGCAGCTCATGGCTTCGGGGTTCAGCTTGTCTGG -----+-----+-----+-----+-----+ TGTTCCGGCTCTGGCTGTCTGAGTACCGGAGCGCGGACTCGAACCGCC	3150	3401	CGCCCTAGCGCGCGCGCTCGGTCACAGCGCGCTCGCGGCTGATTTGCGCG -----+-----+-----+-----+-----+ CGCCGATCCGCGCGCGCGCGAGCCGACGCTGTCCGGCAGCGCGGACTAAGCGGC	3450
	K A E T E Q L M A F G R Q L G R			A * A A R P R T W L G D R S I E G	
3151	GTCCTTCAGTCTCATGACGACCTCTCGACGCTGATCGCGGACAGCGCAG -----+-----+-----+-----+-----+ CAGAGGTCAGGATACTGCTGGACGACCTGCACCTAGCCGCTGTTCGGTC	3200	3451	CGCGGACAGCGCGCATGCGCGCGCTCCAGCGCTCCGCGCGCGCAGAGCGCC -----+-----+-----+-----+-----+ GGCGGTCCGGGCTACGCGCGCGCAGGTTCCGAGCGCGCGGCTTTCGGG	3500
	V F Q S Y D D L L D V I G D K A S			G R L R S A A A D L G G R A L L G	
3201	CACCGCGAGGATACGGCGCGGACACCGCGCGCGCGCGCGCGCGCGCGG -----+-----+-----+-----+-----+ GTGCGGTTCCATGCGCGCGCGCTGTGCGCGCGCGCGCGCGCGGTTCCGCG	3250	3501	GATCTTGCGAGGCTTCGACGCTGCTGATCCGCTGGCGATAGCGGCTCGGGCG -----+-----+-----+-----+-----+ CTAGAACGCTCGGAGCTCGACGAGCTAGGCGACCGCTATCCGCGAGCGCGC	3550
	T G K D T A R D T A A P G P K G G			I K A A K S T S I R Q R Y A E P	
3251	GCCTGATGCGGTCGACAGATGGCGGACGTGGCGGACGATTAACCGCGCC -----+-----+-----+-----+-----+ CGGACTACCGCGGACGCTGTCTACCGGCTGCACCGCGGCTGATATGGCGCGG	3300	3551	CACCTTGGCGGATGCGGCTCCGATTCGCGATAGATAGCGAGCGCGCGG -----+-----+-----+-----+-----+ GTGGACCGGCTACGCGCGAGCGGCTAGCGGCTATCTATGCGTCCGCGCGC	3600
	L M A V G Q M G D V A Q H Y R A			G G Q R I R T G I A R Y I R L A A	

Fig. 7/7

3601	GGATCGACCAAGCGGAGCGCGGCGGAGATCGGAGCGCCCTCGCGGC CGCTAGCTGTGCTCGCGCGCGCGCTAGCGCCCTTCGGGAGCGCGCG	3650	3901	CCACGACCCCGCGGACGCTGGTAGGAAATTCACGACGCTCATCGAGGT GGGTGCTGGGGCGCTGCACCATCCCTTATAGGTCTGCTAGTAGTCCGA	3950
	A I S W A C R P P L H P L G Q R A			G V V G A V R Y S Y E L V D D L S	
3651	CGAGGCATATAGGCTCGCGCGGTCAGCGAGCGGATGATGACGGAT GCTCCGTATTATCCGAGCGCGGCGAGTTCGTCCGCTACTACTGCGTTA	3700	3951	CGGTATTTCGGATCCGCGACATCCATCCGMAACCTTCATCAGGTCCA CGCATTAAGCGCTAGCGCTGTAGGTAGCGCTTTCGGACCTAGTCCAGGT	4000
	S A Y Y P E A A D L L R I I V S			R Y E R D A V D M A F G E I L D	
3701	AGAGCGGTCCGAGGCAACCGACCTCAACCGTCGCCCGCGCTCGGCG TCTCGGCAAGCTTCGTGCGCTGGGAGTTGGCAGCGGGGCGAGCGCG	3750	4001	TGCGCCAAAGGTCCGGMAATCATCCGCGCGGCGGACCTGGGCGAGCGCC AGCGGTTCGAGGCCCTTTATGTACGGCGCGCGCTGACCGCGCTCGCGG	4050
	Y L A D S P V P G E V T A G A E A			M P W L D P F D H R R A V Q R L A	
3751	AGCCAGTCGCGAGCAGATAGCAGCGCCGATCGCGGATCGTCGATCAC TCGGTCAACGCTCGTCTATCGTCGGGGCTACCGCGGTAGCAGCTAGTG	3800	4051	CGMAAGCGCGGAGATCGGCGCGCTCGTCGAGCGCGCGCGCGCTGTC CGCTTCGCGCGCTGTAGCGCGCGGACGACGTCGCGCGCTCGCACAG	4100
	L W D A P L Y C R G I A A D D I V			A F P P S M P G D E H L A A L T D	
3801	GTCCGAGCGGATCTTCGTGAGCTGGAGCGCAGCGCGAGATCGCAGGCG CAGCGCTCGCTACAGCAGTCAACCTTGGCTTCGCGGTCTAGCGTCCGCG	3850	4101	GCGCGCAGCGCGCGCGCGCGCGCTGTGCGTCCGCGCGCGCTCGGGG CGCGCGTCCGCGCGGTGCGCGCGCGGACCCAGCGCGCGCGCGCGCGCG	4150
	D R A I N T L Q F A L G L D C A			A R L A G L R A Q P D G G A E P	
3851	GATCCAGCAGCGGATCGCTCCACCGCCCATCAACCGCGGCGATCATCAG CTAGTCTCGCGTAGCAGGACGTGCGGCTAGTGGCGCGCGCTAGTGC	3900	4151	CAGAACCCATCAGCTGCCCGTCGATCACTCATCCGATCGCTGCACCCAG GTCTTGGTAGTGACCGGCGAGCTAGTGCAGTAGCGGTACGAGCGTGTGC	4200
	R D L V A D D Q V G N V R A M N V			A S G M V Q G D I V D D A H R C W	

Fig. 7/8

4201	GCATAGCATGACCGTATCTCTCGCGATGCCGGGGCGCATCACTTGGC CGTATCTGCTACTGOCATAGAGCGCTACGGCCCGCTAGTGCAGACCG A Y L M V T D E R I G P P M L K A	4250	CGTATGCGCCGACAGTTCTGCTCTCAAAATCGCGGGCGCTCAAGATGGG GCACTACCGCGCTGTCAAGCCAGCACTTTAGCCGCCCGGACTTCTACGCC G H H A S L E T S F D A P S F I R	4501	4550
4251	CGCTGCGGAGCTTTCCGAACCTGCGGATGGCCGCTTCGGAATCG GGGACCGCGCTTCGAACGCTTGGACCGCTTACCGGGAAGCCTTCAGC A Q A F S Q S G Q A I A A E S T	4300	CTGACGGTCAAGTGTGCGGAGTGGCGGATGGCGGGGCTCCAGTTC GACTGCCAGTCCACCAAGCGCTCCAGCCCTACCGGCCCGGAGGTCAGG S V T L H K R L D P I A R R E L E	4551	4600
4301	CGCTCAGATCGGTCATCGGACCGCGCGTCCGACAGCATGACCTGGCCG GGCAGTCTAGCCAGTACGCTCCCGGTCCAGGCTGTGTACTGGACCGGC A T L D T M * A V A L D S L M V Q A <-- grtB	4350	CTCGAAGATGGCTGGGCATACCGCGGGGCTCGGCTTCCCAATCGACAT GAGCTTCTACCGAGCGCTATCGGGCCCGGAGCCGAGGTTAGCTGTA E F I R E A Y G P A E A E W D V	4601	4650
4351	TGGCTTGGCGTCCCAACACACCGCGATCCCGCACCGCATGCGTG ACCGGAACCGGACGCTGTGTGGCCCTACGGCGGTGGGCTTACGCAC T A K A S G V V G P I G A G P H T	4400	CGCGCGCCCGCAGATCGGGAAGGGCGGAGGACGTAATCGGTGGACATC GGCGCGCGGCTCTAAGCGCTTGGCCGCTTCTGCAATTAAGCACTGTAG D A R G L H P V P A L V Y H T S M	4651	4700
4401	CCCGCCCGCAGATGTACAGTTGCGGATCGCGGCTCGCGGTATGCGG GGCGGGGCTGTACATCTTCAAGCCCTAGCCGCCAAGCGCCAAATACGC G A G V I Y F N P I A R D R N H P	4450	CCCTCGGGGGCCAGGCTGGATCGCTCAGCAGGGCGAATGCAAGATACAT GGAGCGCCCGGCTCGGACCTAGCCAGTGGTCCCGCTTACGCTCTATGTA G E P A L S P D T V C P S H L Y N	4701	4750
4451	GGGAAACGAGGGGATTCGTCAGATCGGCTCGACCGGAGAGCGCGCTGC CGCTTGGTCCGCTTACGAGTCTTACCGAGCTGCTCTTCCGGGACG R F H A S Q T L I P E V S F A S	4500	CGAGAAATCGTCCGCGAGGCGTGGCCCGCTTGAATCTGTTCCACCGCC GCTCTTTAGCAGGGCGTCCGACCGGCGAATCTTAGAGCAAGTGGTGG S F D D P L R P G N F I E N V L	4751	4800

Fig. 7/9

4801	CCTTGTATGCGCGGCGCAAGATGACCGCTGTGCTGGCGCAAGTTCTCGGG -----+-----+-----+-----+-----+ CGAATCTGCGCGCGGCTTCTACTGCGACACCAACCGGTCGAAGAGCGCC G K Y R P G F I V S H A L N E P	4850	5101	GCTCGACAGGGCGACCATGCCCCGGAACAGCTGTTGTCGCCGCCCTTG -----+-----+-----+-----+-----+ CGAGCTTGCTCCCGCTGGTACGGGGCGCTGGTCGACCAACAGCGGGGAGC R E F L A V M G A V L Q N T G G K	5150
4851	CCGTTGACAGCGCGCAATGACGACGCAACAGCAATCCAGCGCGCTG -----+-----+-----+-----+-----+ CGCAACCTGTCGCGCTTACGTCGTGCTTGTGCTGTAGCTGGTCGCGAC R K S L G F H L V F L S M S W R Q	4900	5151	CCGAAACAGACCGCGCGCGCGCTTCCAGCGCATGCAATCAGCGCATAGAT -----+-----+-----+-----+-----+ CGCTTGGTCTGCGCGCGCGCGCGCAAGTCCGCTAGCTAGCTGCGTATCTA A F W V G G R R E L A H I L A Y I	5200
4901	CGGTTACAGATCGCGCTTGGTGGCGCGCGGGGGTATGCGCCAGCA -----+-----+-----+-----+-----+ GCCAAGTCTAGCGCGCGCAACACAGCGCGCGCGCCCATACCGGCTCGT R N L I A A K T R G R R T H G L	4950	5201	CGAGCTGTCGAAACAGCGGTTCCGCGCGCAACAGCAGCGCTGTGAAACAGA -----+-----+-----+-----+-----+ GCTCGACCAAGCTTTTCCCAAGGGCGGCTGGTCGCGACACACCTTCTCT S S T S F P N G G V L L T H F S	5250
4951	GGTCCGATAGCTGTGATCAAGTCGCGGTTGCTGGCCACCGTATCCGG -----+-----+-----+-----+-----+ CCAGCGCTATCGACAGCTAGTCAGCGCGCAACGACCGGTGGCATAGCGCG L D R Y S H M V D G N S A V T D A	5000	5251	AGGCTGCCGCAAGATCGCGGTCCTGATGAAACCGCGCAACATCGTGTGG -----+-----+-----+-----+-----+ TCCGACGGCGCTACGCGCGCAGGACCTACTTCCGCGGCTGTACGACACC F A Q R L H P D Q I F R A V M S H	5300
5001	CGCAACTCCCGCCGTCACAGCGGTGACGCCGCTGGCGCATCGCCCTC -----+-----+-----+-----+-----+ CGGTTGACCGCGCGGAGGTGTCGCACTGGCGGCAACCGGCTAGCGGAG R L Q R G D L L T V G T A R D G E	5050	5301	ACGACGGGTATGCTCGAGCGCGCATCAGCGCGCGCGCGGCTTCAGCAT -----+-----+-----+-----+-----+ TGGCTCGCATACGAGCTGCCGCTAGTCGCGCGCGCGCGCGCAAGTCGTA V S R Y A Q L R M L A P A A N L M	5350
5051	GGTTCGATCCCGTCAACCGCGCATTCACGACGACGCTGCGCGCAAGAC -----+-----+-----+-----+-----+ CCACAGCTAGGCGCATCGCCCGGTAGTCTGCTCGCACCGCGGTTCTG T D I R T V R A N L L L T T G G L	5100	5351	CTGCGCCAGCTTCAGGAGGGGCTGTCCGAGCTTCAGATACCCCTCGC -----+-----+-----+-----+-----+ GACCGGTGCAAGTCTCTTCCGCGCACGAGGCTCGAAGTCTATGGGAGCG Q G L K L F P T T G L K L Y G E	5400

Fig. 7/10

```

5401  GATAGACCTCCTCGGGTAATCGTGGAAAGCGCGCATGCCATCGACATCG
      +-----+-----+-----+-----+-----+
      5450  CTATCTGACGAGCGCCATTAACACCTTCGCCCTATCGGTAGCTGTACG
      +-----+-----+-----+-----+-----+
      R Y V E E A Y D H F R R Y G D V D

5451  GCGGATTTAAAGGAGCGGACCTGCCGATCAGCTCGTCTGCTCTTAC
      +-----+-----+-----+-----+-----+
      5500  CCGCTAACTTCTCTCCCTGGACCGCTAGTCGAGCAGCAGCGAAGTG
      +-----+-----+-----+-----+-----+
      A P N F S A V Q R I L E D D D N V

5501  GTATTGMAAGCTCGGGCGCTCGCCCATGTCCAGCCGGTAGAAGGGGAGA
      +-----+-----+-----+-----+-----+
      5550  CATAACTTTCAGCGCGGACGGGGGTACAGTCGGCCATCTTCGGCTCT
      +-----+-----+-----+-----+-----+
      Y E F S R G D A W T L R Y F P S

5551  CCGCAGCAGCGTCAGCTCAGCGTCCATCGCTTGGCGCTCAGGCGCCAC
      +-----+-----+-----+-----+-----+
      5600  GCGCGTCTGTCGACGTGCGAGGTAGCCAACCGCGGACTCCCGGGTG
      +-----+-----+-----+-----+-----+
      V P L L T V D R E M P Q G S L A W

5601  AGCTTCGCGAGCTCTCGGGTGGTCCAGCACCTCGCGCTCGACATCGAA
      +-----+-----+-----+-----+-----+
      5650  TCGAGCGGTCCGACAGCCCGCAGCCAGTGTGGCAGCCCGACGTAGCTT
      +-----+-----+-----+-----+-----+
      L E R L S D P D T V V T P G A D F

5651  GACCTGGCCCTGATCTTCAGACATAGGCGCGCGCGCGCTGTCCG
      +-----+-----+-----+-----+-----+
      5700  CTGACCGGGACTAGCAGTCTGTATCCGCGCGCGCGCGCGCGCGCG
      +-----+-----+-----+-----+-----+
      V H G Q D N W V Y A R G G P K D

5701  GCGCTCGACGATCTGCTGCTGCCGATCGCGCGGATTCAGCGGATCGCA
      +-----+-----+-----+-----+-----+
      5750  CCGGAGCTGCTACACACGAGCTACCGCGGCTAACGTCGCGCTACCGT
      +-----+-----+-----+-----+-----+
      R A E V I T T A I G A S Q L R I A

5751  AGCCAGCCCGCGCGAACTCTGCCGATGACGATGGCGAACTCATGCT
      +-----+-----+-----+-----+-----+
      5800  TCGGTTCCGGCGGCTTTGACCGGCTACTGCTACCGGCTTGAGTACGA
      +-----+-----+-----+-----+-----+
      L A L G G F G A G I V I A S S M <-- crtI
      +-----+-----+-----+-----+-----+
      A

5801  CTCCTCTCAGCAGGGGGGCTTGGGCGAGCAGCGCAGCGCTGCGACAG
      +-----+-----+-----+-----+-----+
      5850  GAGAGGACGTCTCTCCCGCGCAGCGCTGCTGCTGCGGACGCTGTC
      +-----+-----+-----+-----+-----+
      R E Q L L P R E P L C R V A Q S L

5851  CGMAATGGGGCGGCTCGGTCAGCATGCGAAGCGGCTCGGCGCAATGTCA
      +-----+-----+-----+-----+-----+
      5900  GCCTTACCGCGCGCAGGCCACTGCTACGCTTCGCGCAGCGGGTTACAGT
      +-----+-----+-----+-----+-----+
      P I P P R G T V I R L R D A L T

5901  GCGCGCGGCAATAGAGCGCTCATCAGCGCTCGCGCAGCGCGGTAGAAC
      +-----+-----+-----+-----+-----+
      5950  CCGGGGCGGTATCTTCGAGGCTAGTCGCGCAGCGGTCGCGCATCTTG
      +-----+-----+-----+-----+-----+
      L R C A Y F R E I L P Q P L R Y F

5951  CCGTCAGCAGCGGATAGCAGCGTTCGGCGCGCAGCGCGGAGCAGCAT
      +-----+-----+-----+-----+-----+
      6000  GCGACGTGCTCGGCTATCGCTCGCAGCGCGCGCTCGGCGCTTGTCTGTA
      +-----+-----+-----+-----+-----+
      R Q L L R Y R R D P P C G R F L M

```

Fig. 7/11

6001	CGCGTTCAGCAGCCGCAAGAGGGTCCGATCCGCGCGATCGATGGCGG -----+-----+-----+-----+-----+ GGCCAGTCTGCGCGTCTTCCAGCGCTAGCGCGCTAGCTACCGGG	6050	6301	CAGCAGCGCTGCGCCAGCGCGCATCGTCCAGATCGCGCGCGTGGCTGT -----+-----+-----+-----+-----+ GTGCTGCGGACGCGGTGCGCGGTAGCAGGTCTAGCGCGCGCAGCGACA	6350
	R N L L P L F R D R D A R D I A			L S A Q A L A G D D L D G G D S	
6051	AGCGGCGCACGCGCGAGCGGGGAGCGGTCTGTCAGGTCCGCGCGCGG -----+-----+-----+-----+-----+ TCGGCGGTGGCGGCTCCCGCTCGCGCAGTCCAGCGCGCGCGGCGG	6100	6351	AGCGGTATCTCTCATCATGATCGGGTGGACTGAAGGCGCAGCATAG -----+-----+-----+-----+-----+ TCGCGCATAGGAGTCTCTACCGCCACCGCTGACTTCGCGTCTCTATC	6400
	W G R V A R R A S A T T L D R A A			Y R T D E I L I R T P S F P L L Y	
6101	ATGGCATCCGGACTCGCGCGCATAGGCGAGCGAATATCCGGGTGACGG -----+-----+-----+-----+-----+ TACCGTACGCGCTGACCGCGCTATCCGCTCGCTTATAGCGCACTGCGC	6150	6401	ATGAAGCGGTACCGGTCCATCTCGGGAACGCTCCGTCATCATCATCGG -----+-----+-----+-----+-----+ TACTTGGCATGGCGCAGGTAGACGCTTGCCAGCGCAGTACTAGTAGCC	6450
	I A D A V Q A A Y P L S Y G T V P			I F R Y G D M Q P V T A D M I M P	
6151	GTGGAACAGCCCTCCCGCACCGCCAGCGGACCGCCCTGCGCGTGGT -----+-----+-----+-----+-----+ CACCTGTGCGGACGGGGTGGGTGGCGTGGCGGGGACGGGCAACA	6200	6451	GGCTCGACGCGCATCGGGGCGGTGCGTCTCGATCTCGACCGCCACGATT -----+-----+-----+-----+-----+ CGCGAGTGGGTACCGCGTACCGCGCGCAGCAGCTAGAGCTGGCGGTGCTTAA	6500
	H F L G A G L G V P V A G Q A H			R E V G H P A D T E I E V G V F	
6201	CGCGCAGAGCGCTATCGCGTCATGGCGCAGCGCGATGGCGAGTGGC -----+-----+-----+-----+-----+ GCGCGTCTTCGATACCGCATACCGGTGCGCGTACCGTCTCTACGG	6250	6501	TCTGGAACCGCAGCGTCAAGTGGGGGTCTCGACGCGCACCACGGGGTG -----+-----+-----+-----+-----+ AGACCTTTGGGTGCGAGTCCACGCGCCGAGAGCTGCGTGGCGCGAGC	6550
	D R W F G I A D H A L A I P L I G			K Q F G V T L H P T E V A G R A D	
6251	CTTTCGCGCGCATCTCTGCGCGGTCCAGCGCGCGGTGGCGCATATG -----+-----+-----+-----+-----+ GAAGCGCGGTAGAGGACGCGCGAGGTGGGGCGGACCGCGCTATCAG	6300	6551	ATCAGCAGCGCGCTCGATCCGCGAGCGCTCCGTCAGCGTCCCGCGGT -----+-----+-----+-----+-----+ TAGTGGTCCGTGCGAGGTAGCGCTCGCGCAGCGAGTCCGAGCGCGGCA	6600
	R E R R M E Q G T W G R R A A Y D			I V C A A E I R S G D T L T A G T	

Fig. 7/12

6601	ATGTCACGCGTCGGACATCGGTATTCCACGGCCAGATCAGACACCCCTGCA TAGCAGGTCGCACGCCCTGTACGCCATAAGGTGGCTCTAGCTGTGGACGT	6650	6901	ACCGGACAGCCCGCCGCCGATCAGCAGATCATGCGCTCATGTATTGGG TGGCCCTGTCGGGGCGCGCGCTAGTCTAGTACCGAGTACATATAACGC	6950
	D D L T A V H T N W R L D V G Q			G S L G A G A I L L D H S M ←-- GRTY	
6651	GCAGCCGATCAGCGCGCCCGCTCGATCGAGCCATAGCTGTGCTCAGG CGTCGGCTAGTCCGCGCGCGGAGCTAGCTGGTATGGACAGCAGTCC	6700	6951	ATCCGCCCTTCCGGGTCCCTTCAGCAGCGCGCGCGAGCGTTTCAGCTCTG TAGCGCGGAAAGCCGCCAGGAAGTCCGCGCGCGCTCGCAAGTCGAGAC	7000
	L L G I L A G A E I S G Y G T T L			D A G E R D K L L A G S R K L E	
6701	CGCGCGAATGATCGGAAACGGACCTCTCTCATCCGTCCATTCGCGCGG GCCGCTTACCAAGCCCTTTCGGCTGGAGCACTAGCGAGGTAAAGCGCGC	6750	7001	CCTTGAGGCTGTGCAACGAGCGCGCCAGATGAAACCGAAGCTGACGCG CGAATCCGACAGCTGCTGCTCCGCGGCTACTTTGCTTCGAGTGGCTC	7050
	R R S H D P F A V E Q D T W E G R			A K L S D V S P A W I F G F S V C	
6751	ACGAATCGCGCAGCGCGCCAGCCATTCGGGGGAAAGATCCGTCTCGT TGCTTACCCGCTGTCCGCGCGGTCCGTAAAGCCCGCTTTCTAGGCACAGCA	6800	7051	TTCTCGCGCCATGGAACCGCGTCAATGCATCTGTCTGCTGGTAGACCG AAGAGCGCGGTACCTGGCCGACCTAGTAGACACAGCAGCCATCTCGCG	7100
	R I P S L R A L W E P S L D T D			N E R G H V A H H M R H A Q Y V R	
6801	GGCAGCACAGCTGTCTGTCCAGCGCGCGGACCGCGCGCTCGACCATC CCGTCTGTCCACACAGCAGCGCTCCCGCGCTGGCGCGCGAGCTCGTAG	6850	7101	ACGAAGATAGCCCGCGCTTGGGACATAGCCGAAACCGCCAGCCGCCATGCA TGGTTCTATCGCGCGCAACCCCTGTATGCGCTTGGCGTGGCGGCTACGT	7150
	H C S W T H Q D S P G S R A D L M			R L Y G R K P V Y R P P W R G H	
6851	ACGATGCGCGCATCCGCTTCGGGTCCGGACGGCAACCGCCATCAGCGC TGCTACGGCGGTAGCCAGACGCCAGCGCTTCCGCTTGGCGGTAGTCCGG V I R A D P R R D R V A L A I L A	6900	7151	CCAGCCGCTATGCAAGAAATAGTAGATCAGCCCGCTAGCAGGTGACCCCC GGTTCCGCGATAGCTCTTATCTATCTAGTGGGCAATGCTCCACTGGGG V L G D H L F Y Y I L G Y C T V G	7200

Fig. 7/13

7201	ACGCCACGCCACGAGCCAGATCCGACCCCATCCGCGCGATCCGCGAAG	7350	7501	ATGACCAACCCATCCGCGATCCGCGAAGCCATCCGCGAAGCTCCGT	7550
	TGCGCGTCCGTCGTCGCTAGCGTCGCGTACGCGCGCTACGCGTTC			TACTGTCGCGTACGCGCGATCCGCGAAGCTCCGCGAAGCTCCGT	
	V A L W N A L D S G M A G I A F L				
7251	CACGATCGAGATTACCGCGAAGATGACGCCATAGAGTCTCTTCTCGA	7300	7551	TCAAGGCTCATAGCGCGATCATCCGTCGATTCGCGCGCGAAGCGCG	7600
	GTCTAGCTCTAATGCGGCTTCTACTCGCGTATCTCCAGCGAAGAGCT			AGTCCCGAGTATCCGCGTATAGGCACTGTAGGCGCGCGCTCCGCGTC	
	V I S I V A F I V G Y L D N K E				
7301	GCGCGTGTCTGATCCTCTGTCGTGCGATTTATGCCAGCCCGACGCC	7350	7601	GCGCATCAGCGCTTCGTCGCTCGAATATTTATCTTTTCCCGAAGATGG	7650
	CGCGCACCGACCTAGCAGCAGCAGCAGCTAATACGCTCGCGTCCGG			CGCGTAGTCCGCGAAGCGCGACCTTTATATTTACAAAGCGCTTCTACC	
	L A H D H D E D H H S K H W G W G				
7351	AGCGGCCATGCAATGATCCACCGATGGACGGAGTAGCGCGTCACTCCAT	7400	7651	TCGCGCGCGAAGGATTCGAACTCCGACCTTACGGTACCCAAACCGTCCG	7700
	TCCCGCGTACGTACTAGTGGTCCCTACCTGCTCATCCGCGAGTCCGAGTA			AGCGCGCTCTCTCTAGCTTGGAGGCTGGATGCCATGGGTTTGGCAGCG	
	L P G H M I W R H V S Y A T L E M				
7401	CGCGCGACCGTCAAGATGACGGTCAGGATTGCGCGCCAGTCTCATGC	7450	7701	GCTACGAGGCTCGGCTACGCGCCGATCGCGAAGGCTTTAGCGGATTTGT	7750
	GCGCGCTGCGAGTCTACTGCGAGTCTTAACGCGCGGTTTCAGCAGTACG			CGATGCTCCGACCGCGATCGCGGCTGACGCGTTCCGAAATCGGCTAACCA	
	A A V T L I V T L I A A W T S M				
7451	CGCGCGCTTCTCTCATATGACAGCGAAGAGGCTACCTGCGCGCGGTCG	7500	7751	CGCGCAAGGAAAGACTTCTGCTCCAGCGCGAAGCGCATTTGTCGCCATG	7800
	GCGCGCGAAGCACTATCTGCTCTGCTGCTGCTGCTGCTGCTGCTGCTG			GCGCGTTCCCTTTCTGATCAGCGCTCCGCTCTGCGCTAACAGCGGCTAC	
				A D C A L V A N D G M	

Fig. 7/14

```

7801  CCCGATCGCCATCGGCTGACCGGCTTCAGGCGAAGCGGATCCGCTC
      +-----+-----+-----+-----+
7850  +-----+-----+-----+-----+
8101  GCGCGTCTTCGGGGCTCTCCGGAGCTCGACCGGAAACCGGAGGTTTC
      +-----+-----+-----+-----+
8150  CCGCGGAGAGCCCGGACAGCGCTGGAGCTGGGCTTTGGGCTCGCAAG
      +-----+-----+-----+-----+

      A R E P S D A V E V R F G L T E

7851  TCCGCCCGGATTCGAGGACGACAGCGGTCGGGTCCGGATCGCGGA
      +-----+-----+-----+-----+
7900  +-----+-----+-----+-----+
8151  AGCGGGCGCTAAGCTCTCTTCTCGCGCAGCCCGCTAGCGGT
      +-----+-----+-----+-----+

      G P H A M P Q G P K L G L R D A E

7901  CCGCGCGCGCGGAA TGGGCTCTGTCAGCGCGCGCGGCAATTCCGCTGG
      +-----+-----+-----+-----+
7950  +-----+-----+-----+-----+
8201  GCGCGCGCGGCTTACCGCAGAGCAGGTCCCGCGGCTACGCCACG
      +-----+-----+-----+-----+

      V A A G P I P T E D L P R A N R H

7951  ATGTGGCGATGACCGCGGTTTCATCCCGAAGACCA TGTCCAGCGGGAT
      +-----+-----+-----+-----+
8000  +-----+-----+-----+-----+
8251  TACACCGGCTACTGCGGCCAAGTAGGGTTTCTGGTACAGGTCCGCGCTA
      +-----+-----+-----+-----+

      I H R I V G T E D A F V M D L P I

8001  CAGTGTGTTCCGATCCAGAACGACACCGGCTGGGGGATTCGTAGTGA
      +-----+-----+-----+-----+
8050  +-----+-----+-----+-----+
8301  GTACACAA CGGCTAGGTCTTCTGTGCGCAGCCCGCTAAGCATCTACT
      +-----+-----+-----+-----+
8350  CCTCGTTCCGCTCATGCAAGCGCCAGGTCCCATGCCGGATCTCGCGGnnC
      +-----+-----+-----+-----+
      GGAGCAAGCGCCAGTACGTCGCGGTCCAGGGTAGCGGCTAGACCGGChnG
      +-----+-----+-----+-----+

      R T G T M <-- orf-16

8051  ACAGATTCCGGTGC CGCGAGCGAGCTCTTGGGAA CATCAGCCCGTGC
      +-----+-----+-----+-----+
8100  +-----+-----+-----+-----+
8351  TACTCGGCGCGGCTGGAGCTGTGCGCTCGGCTCTAGCGGAGCGGCTA
      +-----+-----+-----+-----+
      FL M C T G A P L E K R F M L G Q

```

Fig. 7/15

8401	CACGAGTCGAGAGCCGGAGTGAAGGAGCACCTCGATATGGATGAACA -----+-----+ CTGCTCCAGGCTCTTGGGCTTACTGGCTCGTGGAGCTATACCTACTTGT	8450
8451	CCTCCTCGGGTGGCCGAAAGATGTTGGCGAACCGGGAAGAGCCCTTGGC -----+-----+ GCAGAGCCCGACCGGCTTCTACAAACCGCTTGGGCTTTTCGGGAAACCG	8500
8501	CTTGTGAAACCACTTGACCGCGCGCGGAGCGAGCGGCAATTCGATG -----+-----+ GAACACCTTGTGAACCTCGCGCGCGCTCGGTCGCGTnnGCCAGGTCTAC	8550
8551	CTCGATCACCTCGGCATCCAGATCGGCGATnGGGGGTGnGnGTCGCTTT -----+-----+ GAGCTAGTGGACCGGTAGGTCTAGCCGCTAnCCCCCACnGnCACGGA	8600
8601	CnnnCGGTTCCATCGACAGGACCTC -----+-----+ GnnnGCCAAGCTAGCTCTCTGGAG	8625

Fig. 8

1 MTPKQQFPLR DLVEIRLAQI SGQFGVVSAP LGAAMSDAAL SPGKRFR AVL  
51 MLMVAESSGG VCDAMVDAAC AVEMVHAASL IFDDMPCMD D ARTRRGQPAT  
101 HVAHGEGRAV LAGIALITEA MRILGEARGA TPDQRARLVA SMSRAMGPVG  
151 LCAGQDLDLH APKDAAGIER EQDLKTGVLF VAGLEMLSII KGLDKAETEQ  
201 LMAFGRQLGR VFQSYDDL D VIGDKASTGK DTARDTAAPG PKGGLMAVGQ  
251 MGDVAQHYRA SRAQLDELMR TRLFRGGQIA DLLARVLP HD IRRSA

Fig. 9

1 MDTLTATSEA AIAQGSQSFA QAAKLMPPGI REDTVMLYAW CRHADDVIDG  
51 QVMGSAPEAG GDPQARLGAL RADTLAALHE DGPMSPFFAA LRQVARRHDF  
101 PDLWPMDLIE GFAMDVADRE YRSLDDVLEY SYHVAGVVGV MMARVMGVQD  
151 DAVLDRACDL GLAFQLTNIA RDVIDDAAIG RCYLPADWLA EAGATVEGPV  
201 PSDALYSVII RLLDAAEPYY ASARQGLPHL PPRCAWSIAA ALRIYRAIGT  
251 RIRQGGPEAY RQRISTSKAA KIGLLARGGL DAAASRLRGG EISRDGLWTR  
301 PRA

**Fig. 10**

1 MSSAIVIGAG FGGLALAIRL QSAGIATTIV EARDKPGGRA YVWNDQGHVF  
 51 DAGPTVVTD P DSLRELWALS GQPMERDVTL LPVSPFYRLT WADGRSFEYV  
 101 NDDDELIRQV ASFNPADV DG YRRFH DY AEE VYREGYLKLG TTPFLKLGQM  
 151 LNAAPALMRL QAYRSVHSMV ARFIQDPHLR QAFSFHTLLV GGNPFSTSSI  
 201 YALIHALLERR GG VWF AKGGT NQLVAGMVAL FERLGGTLLL NARVTRIDTE  
 251 GDRATGV TLL DGRQLRADTV ASNGDVMHSY RDLLGHTRRG RTKAAILNRQ  
 301 RWSMSLFVLH FGLSKRPENL AHHSVIFGPR YKGLVNEIFN GPRLPDDFSM  
 351 YLHSPCV TDP SLAPEGMSTH YVLAPVPHLG RADVDWEAEA PGYAERIFEE  
 401 LERRAIPDLR KHLTVSRIFS PADFSTELSA HHGSAFSVEP ILTQSAWFRP  
 451 HNRDRAIPNF YIVGAGTHPG AGIPGVVGSA KATAQVMLSD LAVA

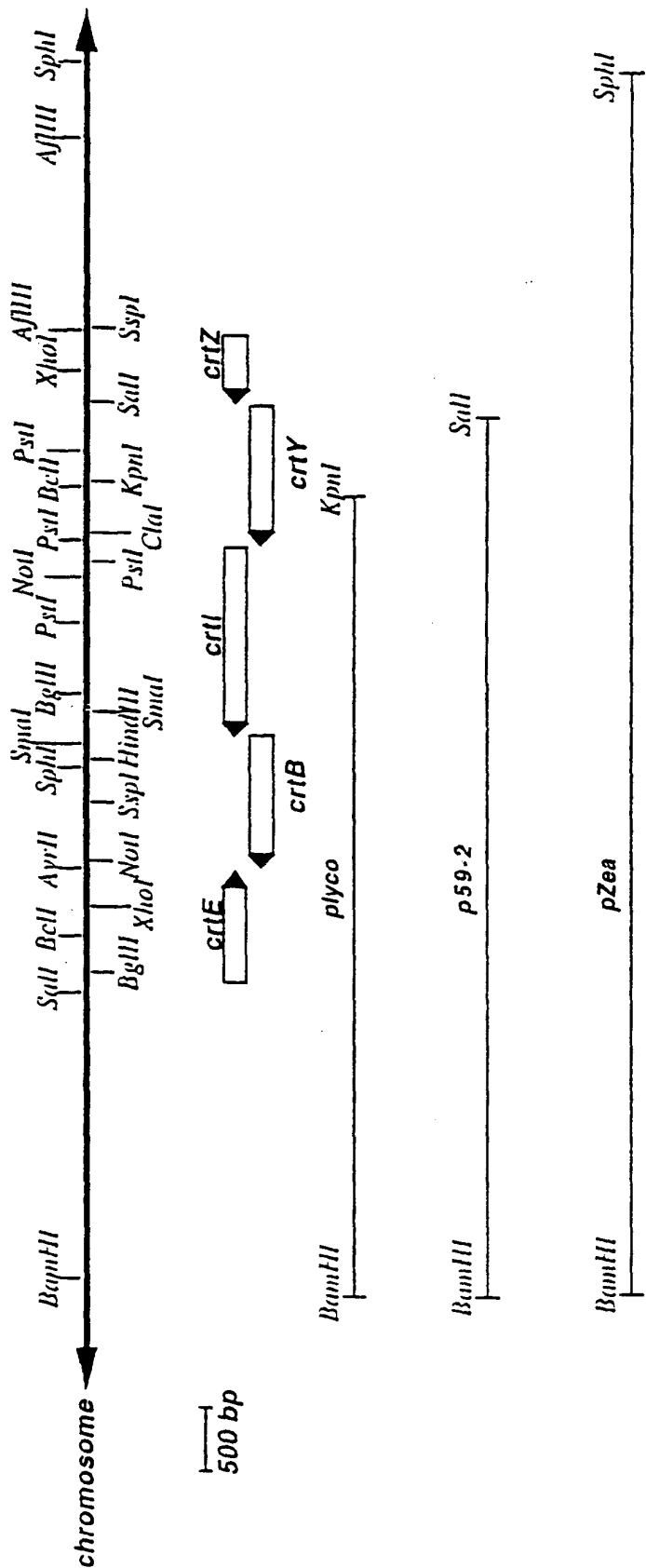
Fig. 11

1 MSHDLLIAGA GLSGALIALA VRDRRPDARI VMLDARSGPS DQHTWSCHDT  
51 DLSPEWLARL SPIRRGEWTD QEVAFPDHSR RLTTGYGSIE AGALIGLLQG  
101 VDLRWNTHTVA TLDDTGATLT DGSRIEAAACV IDARGAVETP HLTVGFGKFV  
151 GVEIETDAPH GVERPMIMDA TVPQMDGYRF IYLLPFSPTR ILIEDTRYSD  
201 GGDLLDGALA QASLDYAARR GWTGQEMRRE RGILPIALAH DAIGFWRDHA  
251 QGAVPVGLGA GLFHPVTGYS LPYAAQVADA IAARDLTTAS ARRAVRGWAI  
301 DRADRDRFLR LLNRMLFRGC PPDRRYRLLO RFYRLPOPLI ERFYAGRLLT  
351 ADRLRIVTGR PPIPLSQAVR CLPERPLLQE RA

Fig. 12

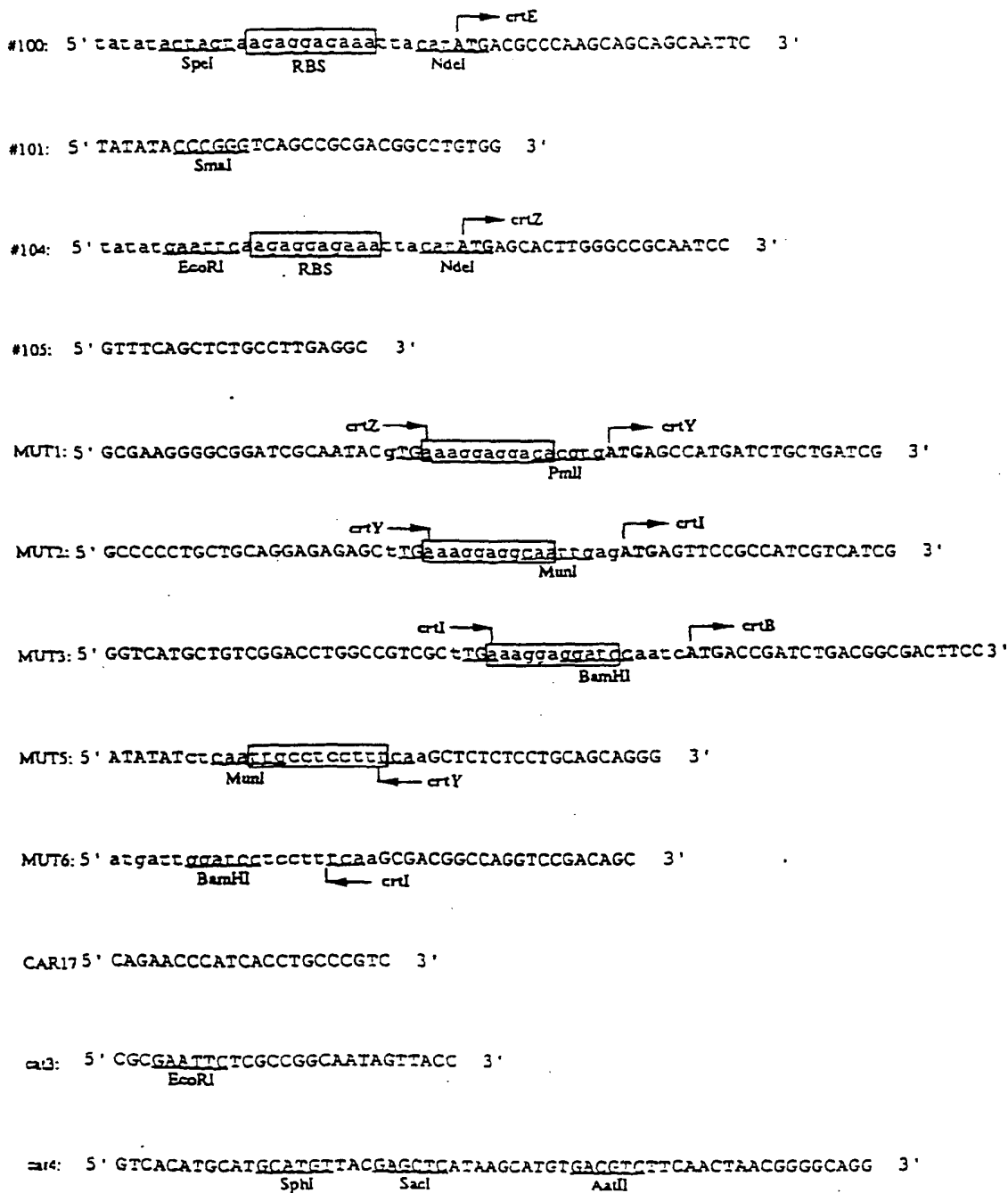
1 MSTWAAILTV ILTVAAMELT AYSVHRWIMH GPLGWGWHKS HHDEDHHDHAL  
51 EKNDLYGVIF AVISIVLFAI GAMGSDLAWW LAVGVTCYGL IYYFLHDGLV  
101 HGRWPFRIYVP KRGYLRRVYQ AHRMHHAVHG RENCVSFGFI WAPSVDSLKA  
151 ELKRSGALLK DREGADRNT

Fig. 13



construct	<i>crtE</i>	<i>crtB</i>	<i>crtI</i>	<i>crtY</i>	<i>crtZ</i>	carotenoid
<i>pLycO</i>	+	+	+	-	-	LYCOPENE
<i>p59-2</i>	+	+	+	+	-	$\beta$ -CAROTENE
<i>pZea 4</i>	+	+	+	+	+	ZEAXANTHIN

Fig. 14



HindIII AflII XbaI EcoRI  
CS1: 5' AGCTTGGATCCTTAAGTACTCTAGAGTTAAACG 3'  
CS2: 3' ACCTAGGAATTTCATGAGATCTCAAATTTGCCTAA 5'

ScaI PmeI  
MUT7: 5' TCGACCCCTAGGCACGTGACGGCTCAATTGGATCCGCATGCCAAGCTT 3'  
MUT8: 3' GGGATCCGTGACGTGCGCAGTTAACCTAGGCGTAGCGTTTCGAACTAG 5'

MunI SphI  
MUT9: 5' gtgtcctccctttcacgatatcgcatccggcccccttcggcggtccttcagcaggcgccgcggcggttcagactctgcccttgaggctg 3'  
MUT10: 3' cacaggaggaaagtgcataacgctaggcggggaaggcccaggaaagtcgtcgcgcggggctcgcaaatgctcgagacggaaactccgacagct 5'

RBS ← criz

Sall AvrII MluI BamHI HindIII  
MUT11: 5' TAAGANAACCctcccttTA 3'  
MUT12: 3' TCCTTGggaggaaaTGATC 5'

Spel  
RBS

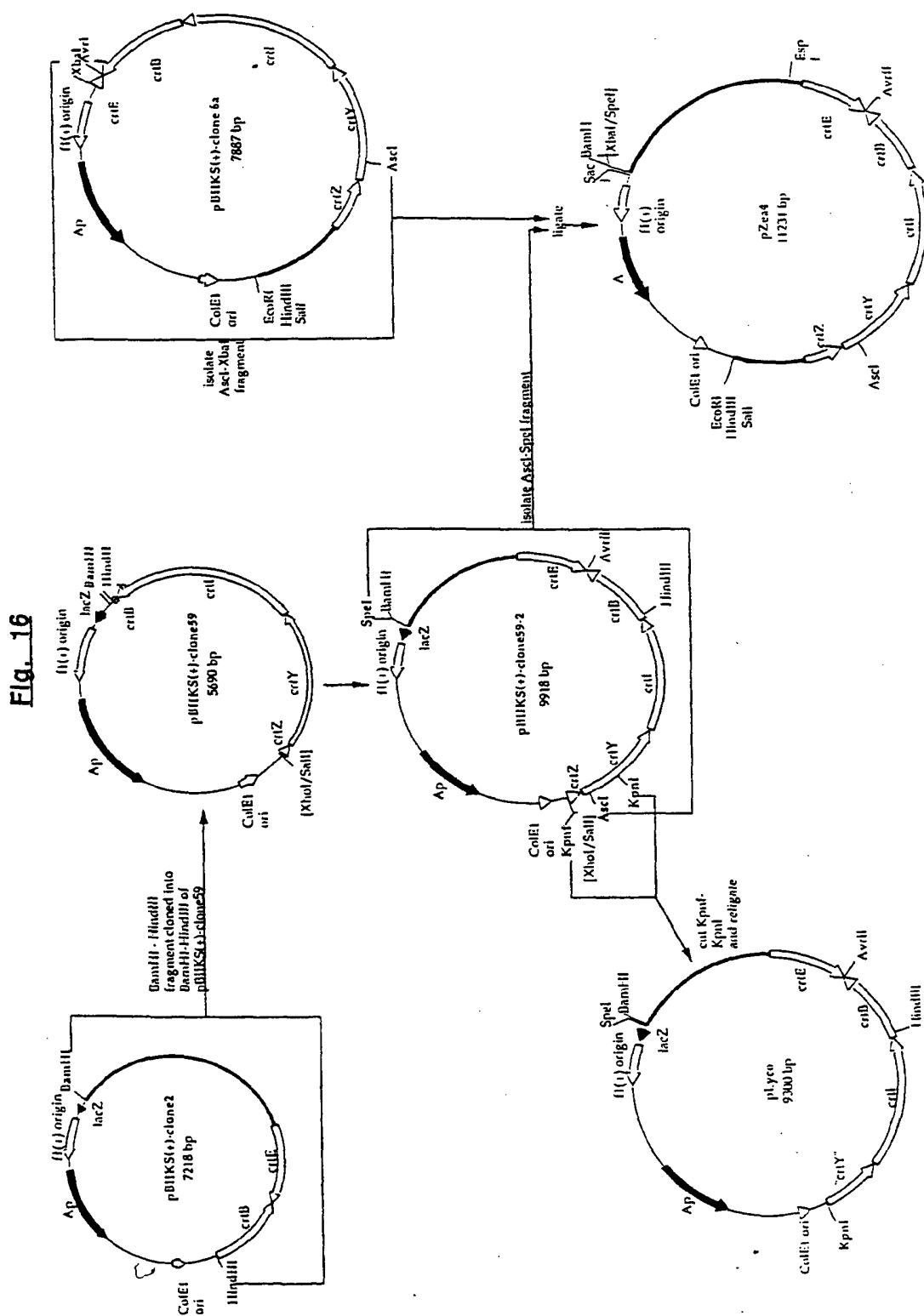
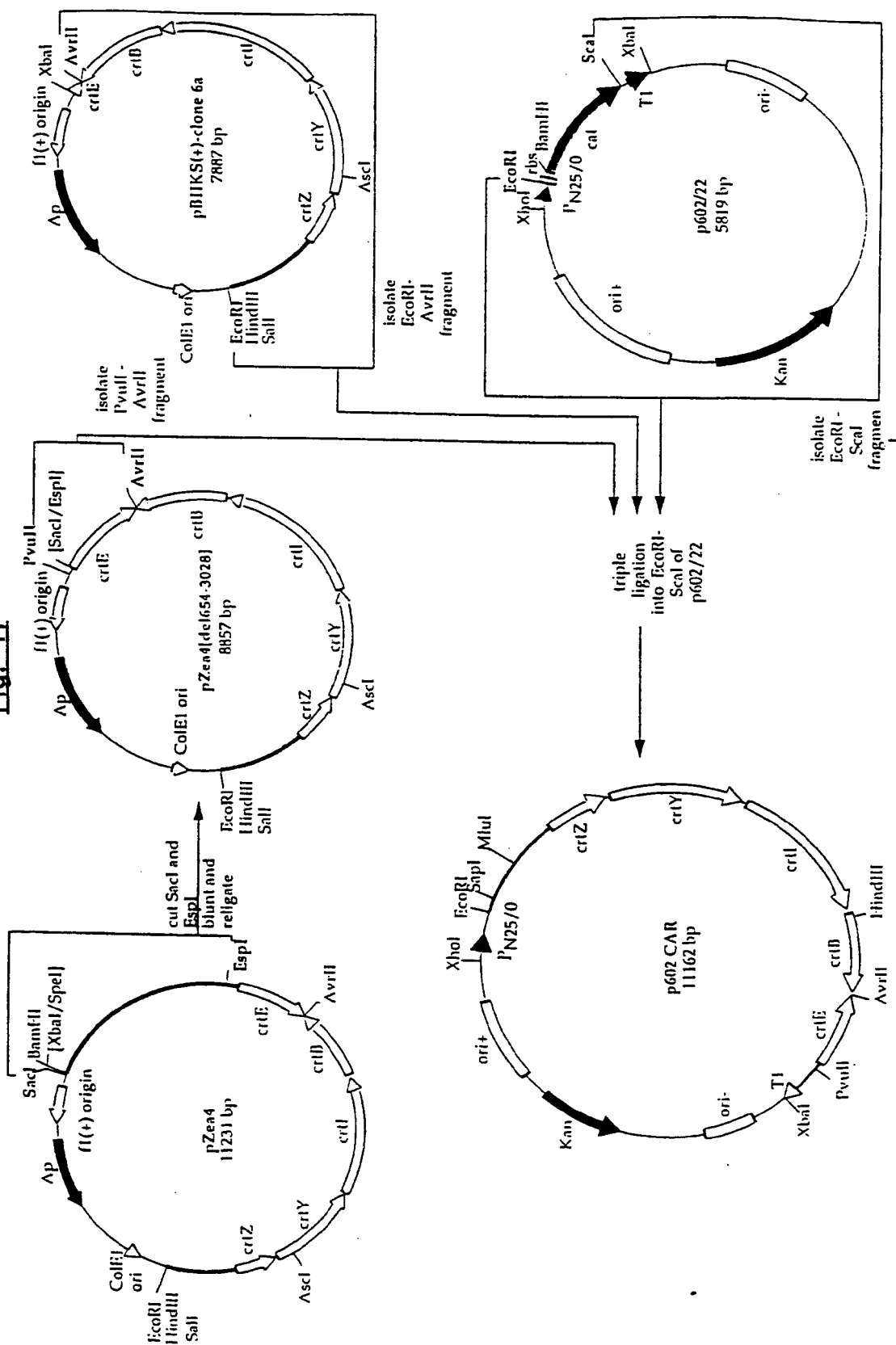


Fig. 17





[illegible]

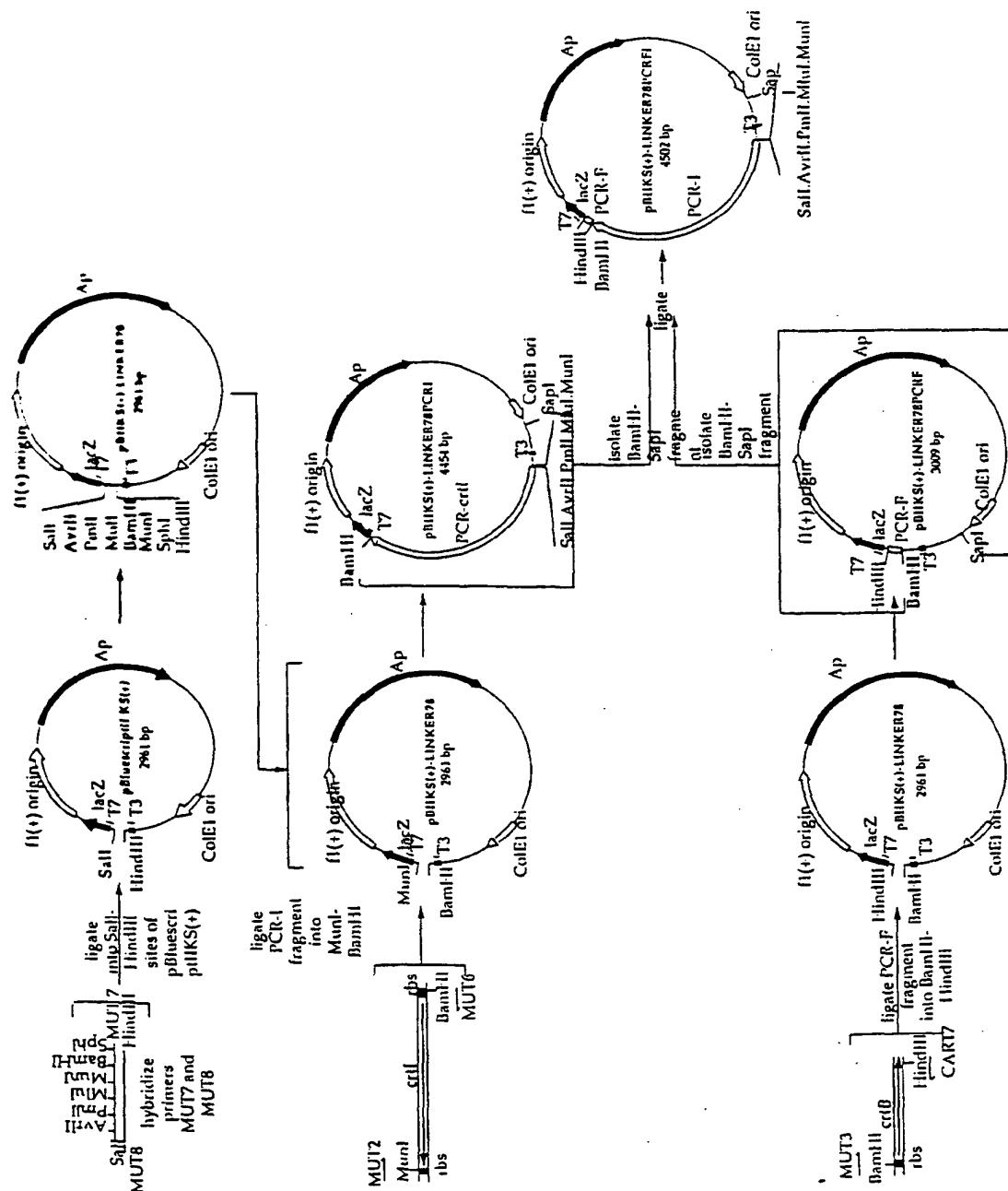


Fig. 20/2

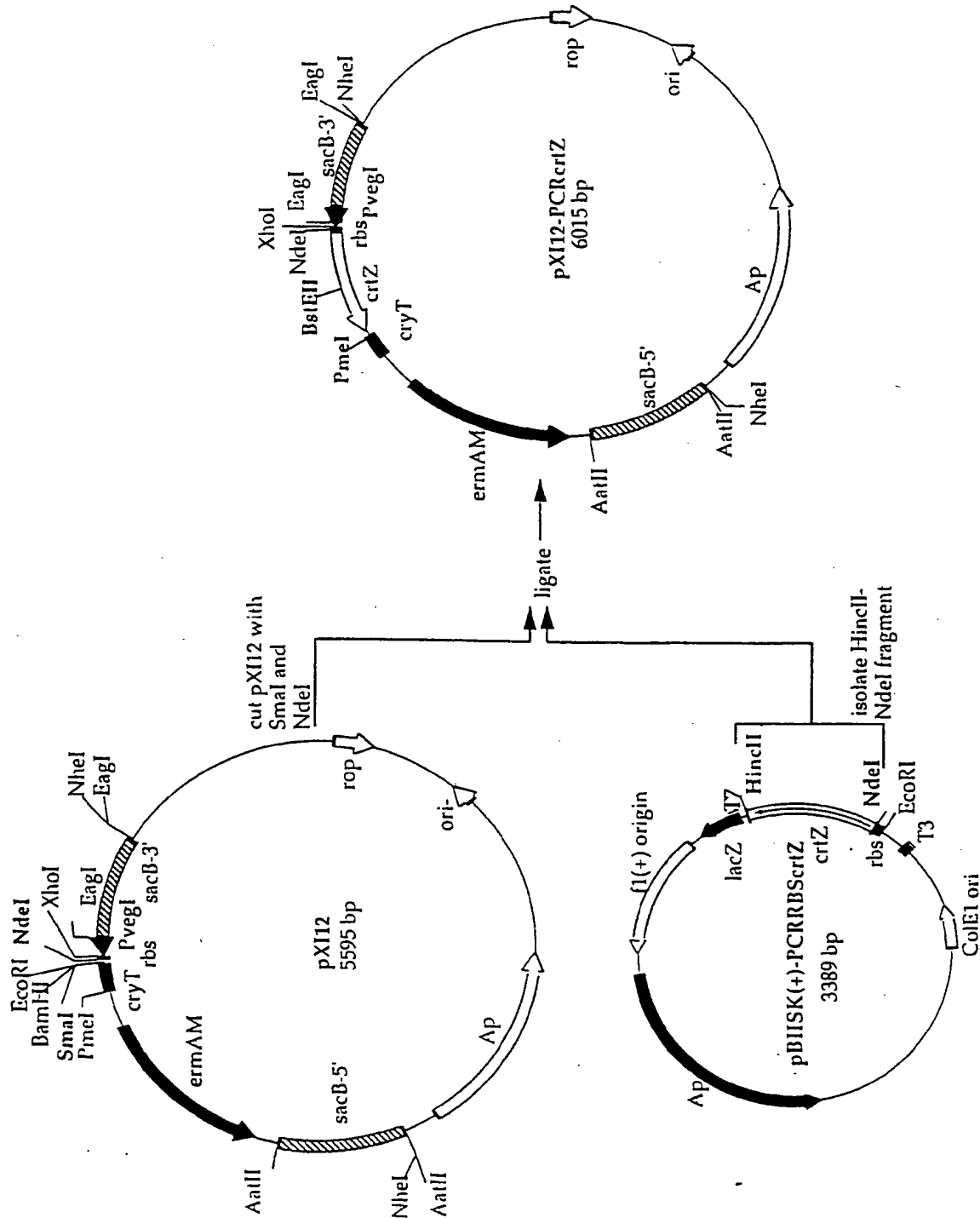




Fig. 20/4

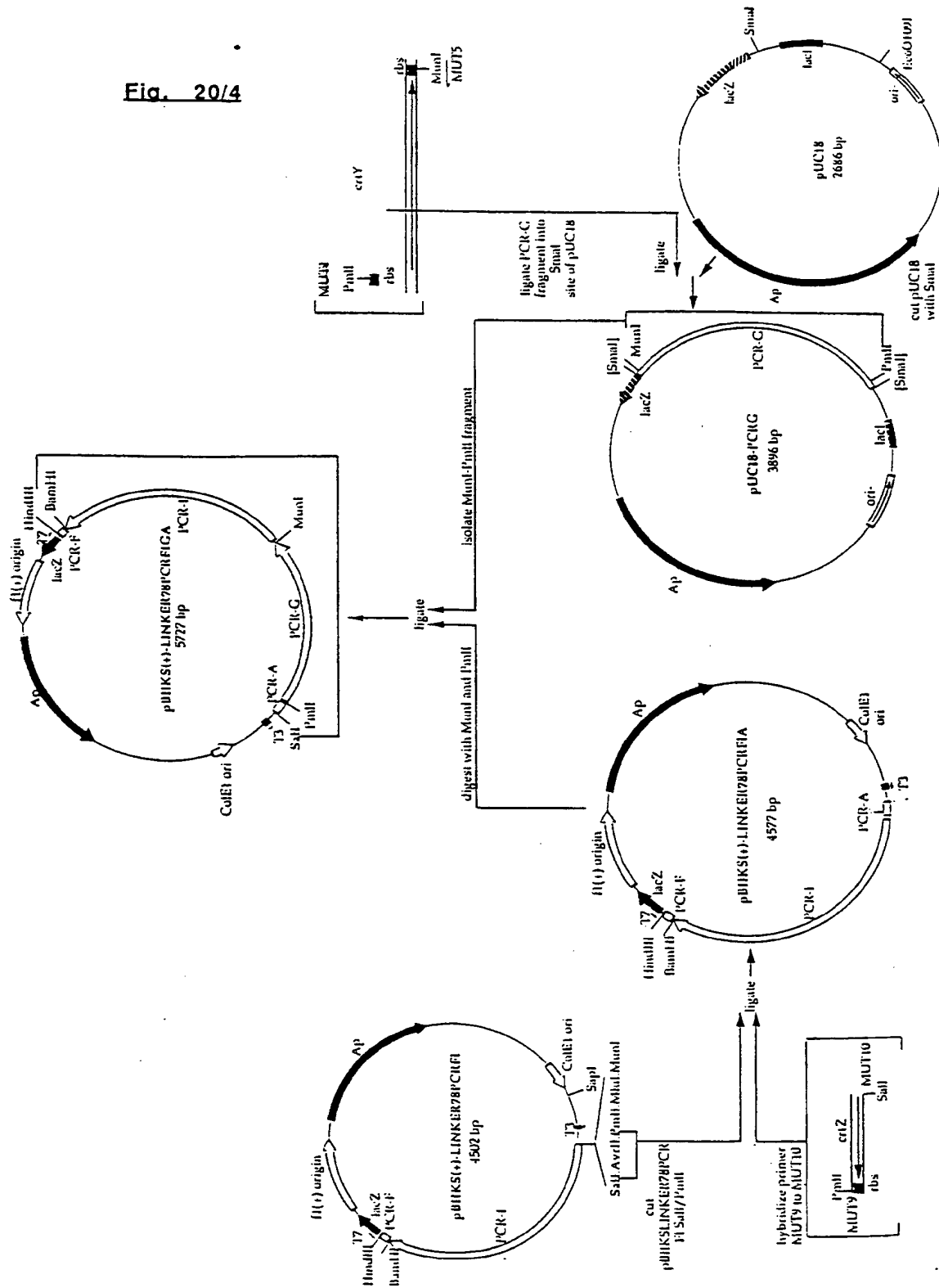
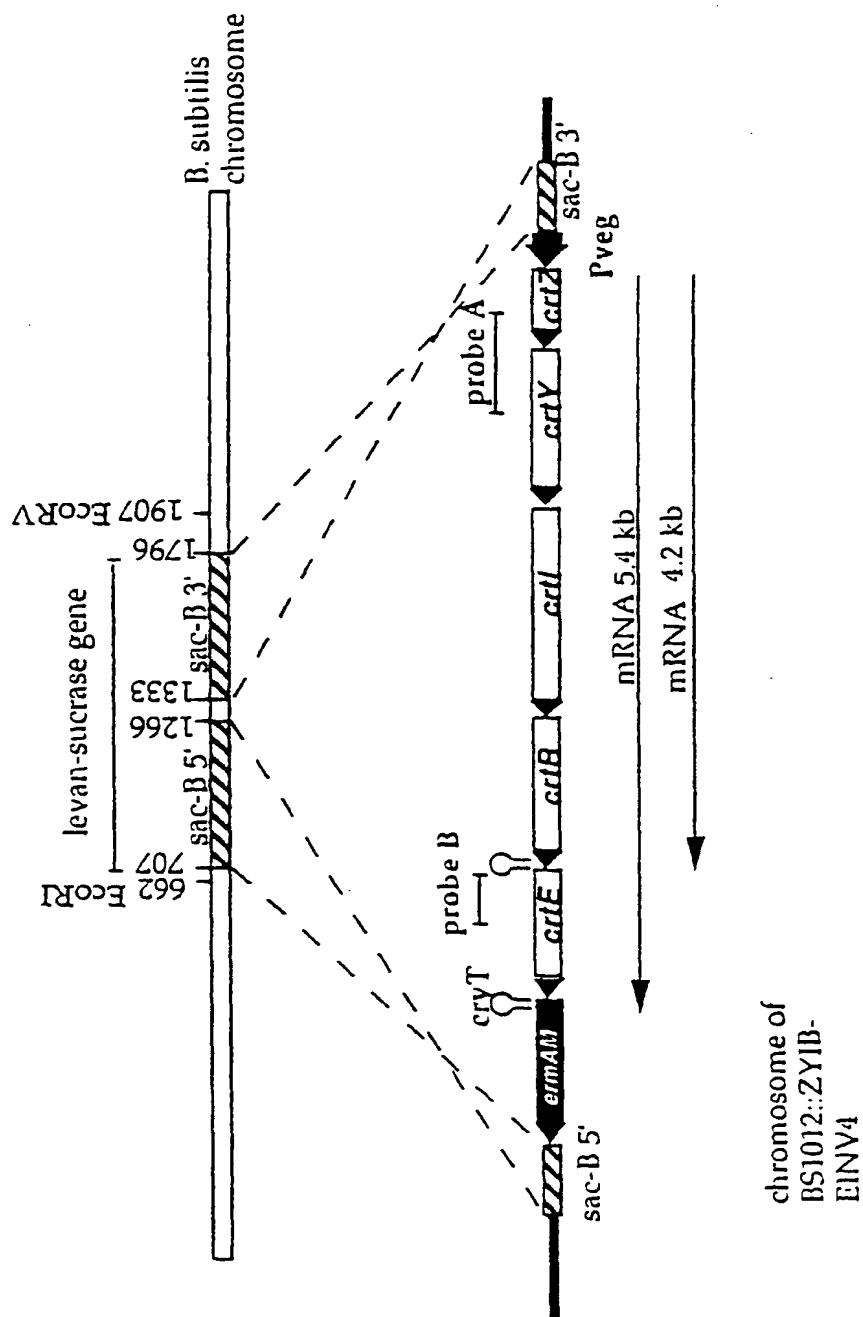


Fig. 21/1



chromosome of  
BS1012::ZYIB-  
EINV4

Fig. 21/2

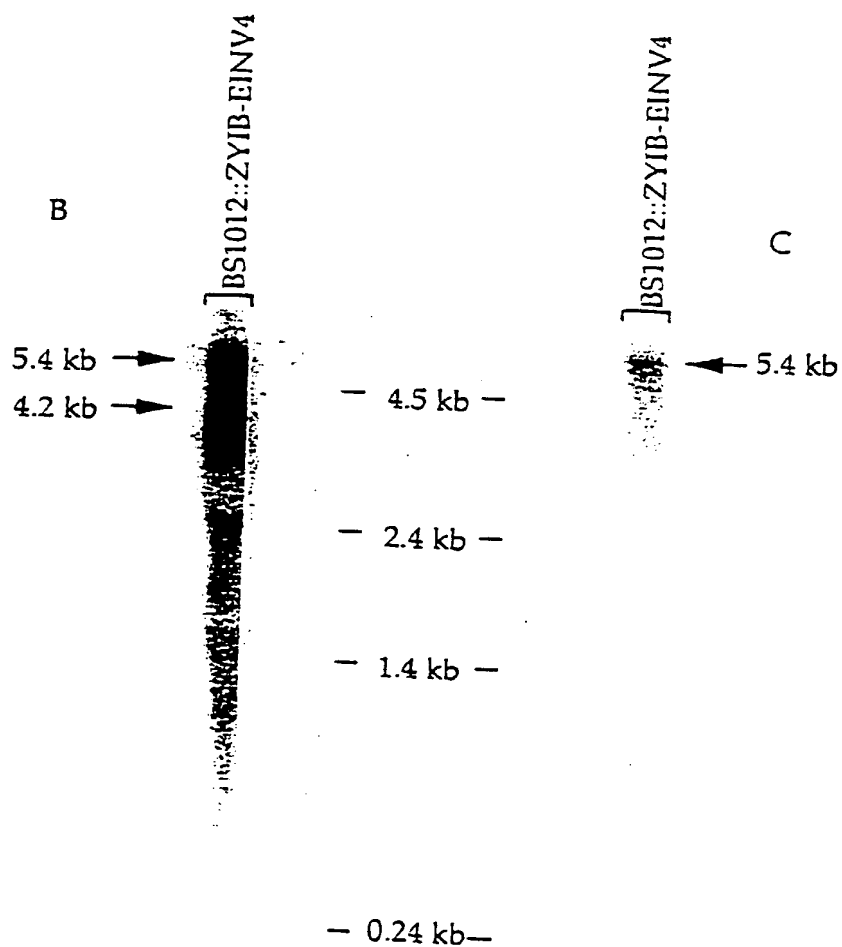


Fig. 22

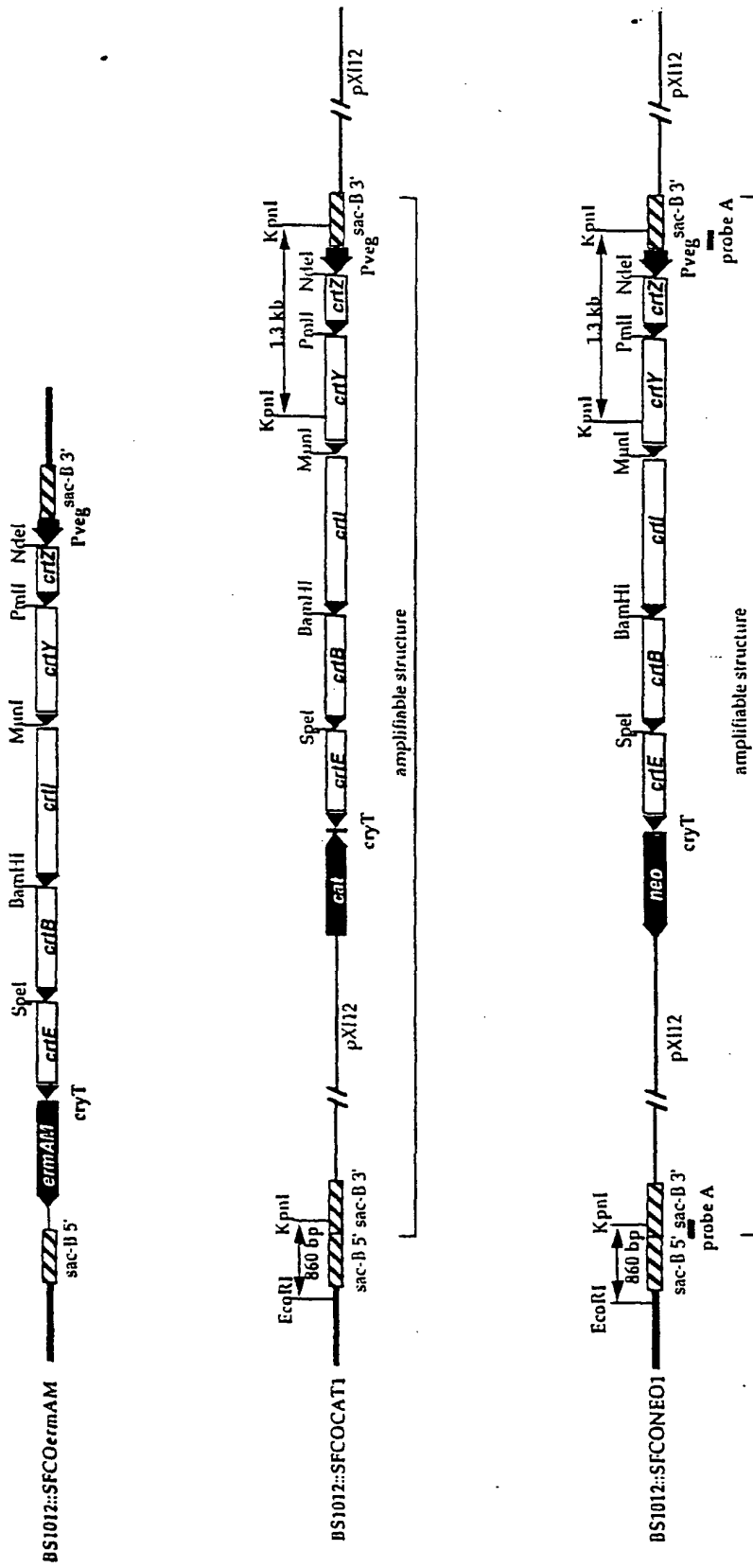


Fig. 23

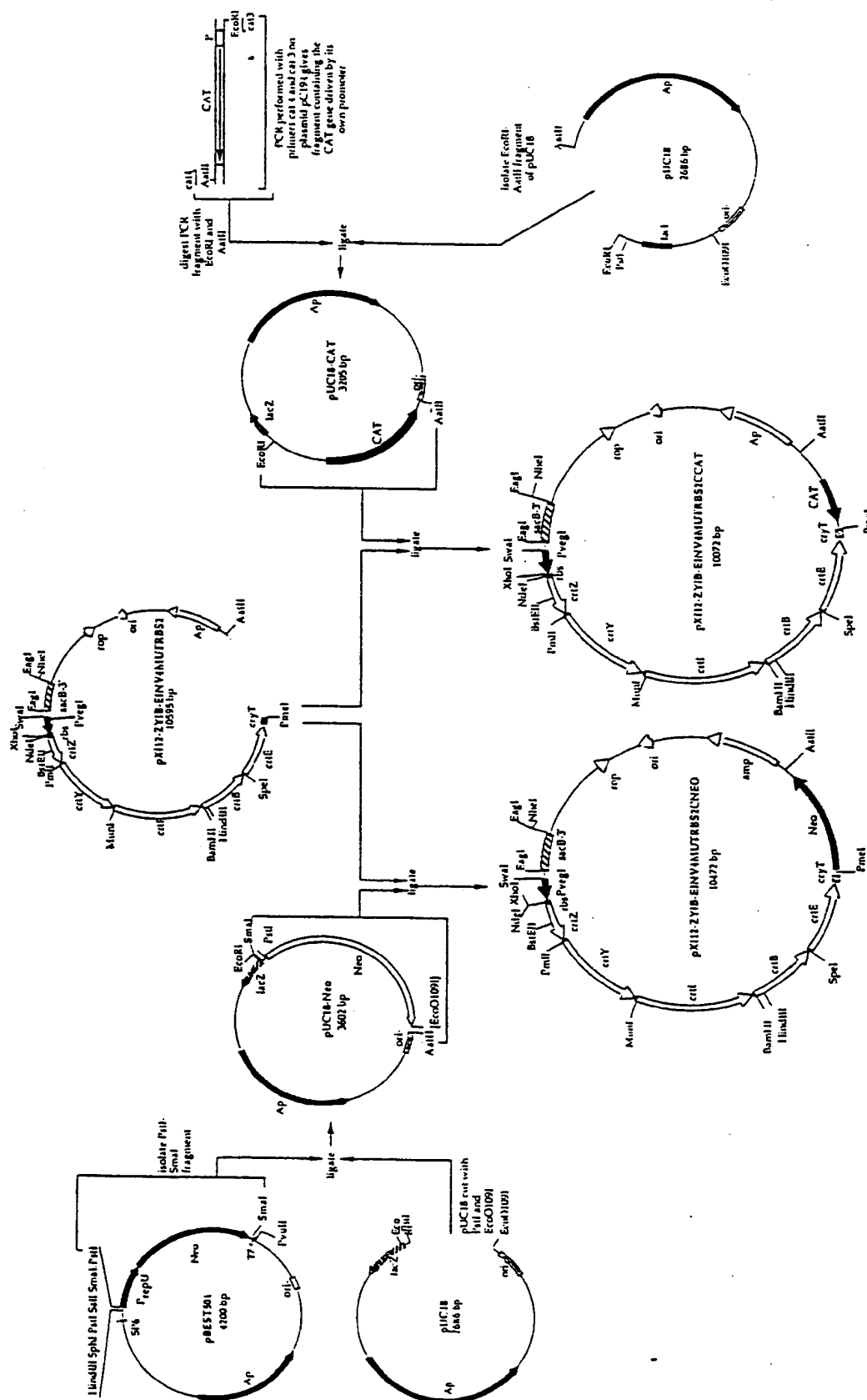


Fig. 24/1

```

CTAAATTGTAAGCGTTAAATATTTTGTGTTAAATTCGGCTTAAATTTTGTGTTAAATCAGCTC
1 ----- 60
GATTTAACATTCCGAATTATAAAACAATTTTAAAGCGCAATTTAAAAACAATTTAGTCGAG
-----
ATTTTTTAACCAATAGGCGGAAATCGGCAAAATCCCTTATAAATCAAAAGAATAGACCGA
61 ----- 120
TAAAAAATTGGTTATCCGGCTTTAGCCGTTTAAAGGAATATTTAGTTTCTTATCTGGCT
-----
GATAGGGTTGAGTGTGTTCCAGTTTGGAAACAAGAGTCCACTATTAAAGAACGTGGACTC
121 ----- 180
CTATCCCAACTCACAACAAGGTCAAACTTGTCTCAGGTGATAATTTCTTGCCACTGAG
-----
CAACGTCAAAGGGCGAAAAACCGTCTATCAGGGCGATGGCCCACTACGTGAACCATCACC
181 ----- 240
GTTGCAGTTTCCCGCTTTTGGCAGATAGTCCCGCTACCGGGTGATGCACCTTGGTAGTGG
-----
CTAATCAAGTTTTTTGGGGTCGAGGTGCCGTAAAGCACTAAATCGGAACCTTAAAGGGAG
241 ----- 300
GATTAGTTCAAAAAACCCAGTCCACGGCATTTCTGTGATTAGCCTTGGGATTCCCTC
-----
CCCCGATTTAGAGCTTGACGGGGAAGCCGGCGAACGTGGCGAGAAAGGAAGGGAAGAA
301 ----- 360
GGGGGCTAAATCTCGAACTGCCCTTTCCGGCGCTTGACCGGCTCTTCTCTCCCTTCTT
-----
AGCGAAAGGAGCGGGCGCTAGGGCGCTGGCAAGTGTAGCGGTACCGTGCAGCTAACCC
361 ----- 420
TCGCTTTCCTCGCCCGGATCCCGCGACCGTTCACATCGCCAGTGGGACGGCGCATTTGGTG
-----
CACACCCCGCGCGCTTAATGCCCGCTACAGGGCGCGTCCCATTCGCCATTAGGGTCCG
421 ----- 480
GTGTGGGCGGCGGAATTACGGCGCGATGTCCCGCGCAGGGTAAGCGGTAAGTCCGACGC
-----
CAACTGTGTGGGAAGGGCGATTCGGTGGGGCTCTTTCGCTATTACGCCAGCTGGCGAAAGG
481 ----- 540
GTTGACAACCTTCCCGCTAGCCACGCCCGGAGAAGCGATAATGCGGTGACCGCTTCC
-----
GGGATGTGCTGCAAGGGCGATTAAAGTTGGGTAACGCCAGGGTTTTCCAGTCACGACGTTG
541 ----- 600
CCCTACAGGACGTTCCGCTAATCAACCCATTGCGGTCCCAAAGGGTCAGTGCTGCAAC
-----
TAAACGACGGCCAGTGAGCGCGGTAATACGACTCACTATAGGGCGAATTGGAGCTCCA
601 ----- 660
ATTTTGCTGCGGTCAGTCCGCGCATTTATGCTGAGTGATATCCCGCTTAACCTCGAGGT
-----
CCGCGGTGGCGGCGCTCTAGTGATCCCGCGCTGGCGGTTCCGGATCAGCAGCCGCCCT
661 ----- 720
GGCGCCACCGCGCGGAGATCACCTAGGCGCGGACCGGCAAGCGCTAGTCTGCGCGGGA
-----
TGCGGATCGGTGAGCATCATCCCCATGAACCGCAGCGCACGACGCGCGCGCCCGAGA
721 ----- 780
ACGCCCTAGCCAGTCTAGTAGGGGTACTTGGCGTCCCGTGCTGCGTCCGCGCGGGGTCT
-----
TCGGGGCGGTCCAGCACGGCATGCGCCATCATCGCGAAGGCCCCCGGCGGATGGGGCGC
781 ----- 840
AGCCCGCGCAGGTCTGTCCGTACGCGGTAGTAGCGCTTCCGGGGGCGCGGTACCCCGCG
-----
GTGCCCCATTCGAAGAACTCGCAGGCTGTCCGCTGCGCAAGGTGCGCGCAGATCGCGCGC
841 ----- 900
CACGGGTAAGGCTTCTTGAGCGTCGGACAGGCGACGCGTTCCAGCGCGGTCTAGCGCGGC
-----
TATTCGGATGCAAGTACGGGCGCGATGCGGCTGGGCGCGCGCTGCCCGCGCGCCACCAGC
901 ----- 960
ATAAGGCTACGTCACTGCCCGGGCTACGGCGACCGCGCGGACGGGGCGCGGTGGTTCG

```

Fig. 24/2

```

961 GCATCGGCACGAAACCTTCCGAGATGATGTGCTGATCCATGGCCCGTCATTGCAAAACC
-----+----- 1020
CGTAGCGCGTGCTTGGGAAGGCTCTACTACACGACTAGGTACCGGGCAGTAACGTTTTGG

1021 GATCACCGATCCTGTGCGGTGATGGCATTGTTTGCAATGCCCGAGGGCTAGGATGGCCG
-----+----- 1080
CTAGTGGCTAGGACAGCGCACTACCGTAACAAACGTTACGGGGCTCCCGATCCTACCGCG

1081 GAAGGATCAAGGGGGGGAGAGACATGGAAATCGAGGACGGGTCTTTGTCTCACGGGCG
-----+----- 1140
CTTCCTAGTTCCCGCCCTCTCTGTACCTTTAGCTCCCTGCCAGAAACAGCAGTGCCCGC

1141 CCGCATCGGGTCTGGGGGGCGGCTCGGCGCGGATGCTGGCCCAAGGCGGCGCGAAGGTCS
-----+----- 1200
GGCGTAGCCCAAGACCCCGCGGAGCGCGCCTACGACCGGTTCCGCGCGGCTTCCAGC

1201 TGCTGGCCGATCTGGCGGAACCGAAGGACGCGCCCCGAAGGCGCGGTTACGCGGCGCTGCG
-----+----- 1260
ACGACCGGCTAGACCGCCTTGGCTTCTGCGCGGGCTTCCGCGCAAGTGCGCGGACCGC

1261 ACGTGACCGACGCGACCGCTGCGCAGACGGCCATCGCGCTGGCGACCGACCGCTTCGGCA
-----+----- 1320
TGCCTGGCTGCGCTGGCGACGCGTCTGCGCGGTAGCGCGACCGCTGGCTGGCGAAGCCGT

1321 GGCTGGACGGCCTTGTGAAGTGGCGGGCATTCGCGCCGGCCGAACCGATGCTGGGCCGCG
-----+----- 1380
CCGACCTGCCGGAACACTTGACGCGCCCGTAGCGCGGCGGCTTGCTACGACCCGGCGC

1381 ACGGGCCGCATGGACTGGACAGCTTGCCTGTGCGGTACGATCAACCTGATCGGCAGCT
-----+----- 1440
TGCCCGCGGTACCTGACCTGTGAAACGGGCACGCCAGTGCTAGTTGGACTAGCCGTGCA

1441 TCAACA TGGCCCGCCTTGACGCGGAGCGGATGGCCCGGAACGAGCCCGTCCGGGGCGAGC
-----+----- 1500
AGTTGTACCGGGCGGAACGTGGGCTCCGCTACCGGGCCTTGCTCGGGCAGGCCCCGCTCG

1501 GTGGCGTGATCGTCAACACGGCCTCGATCGCGCGCAGGACGGACAGATCGGACAGGTGG
-----+----- 1560
CACCGCACTAGCAGTTGTGCCGAGCTAGCGCCGCGTCTGCTGTCTAGCCTGTCCAGC

1561 CCTATGCGGCCAGCAAGGCGGGCGTGGCGGGCATGACGCTGCCGATGGCCCGCGACCTTG
-----+----- 1620
GGATACGCCGGTCTGTTCCGCCCCGACCGCCCGTACTGCGACGGCTACCGGGCGCTGGAAC

1621 CGCGGCACGGCATCCGCGTCTGACCATCGCGCCCGGCATCTTCGCAACCCGATGCTGG
-----+----- 1680
GCGCGCTGCCGTAGGCGCAGTACTGGTAGCGCGGGCGTAGAAGCGGTGGGGCTACGACC

1681 AGGGGTGCGCGCAGGACGTTGAGGACAGCCTGGGCGCGGCGGTGCCCTTCCCTCGCGGC
-----+----- 1740
TCCCGACGGCGTCTGCAAGTCTGTGGAACCGCGCCGCCACGGGAAGGGGAGCGCCG

1741 TGGGAGAGCGCTCGGAATACGCGGCGCTGTTGCACCACATCATCGCGAACCCCATGCTGA
-----+----- 1800
ACCTCTCGGCAGCCTTATGCGCCGCGACAACGTGGTGTAGTAGCGCTTGGGGTACGACT

1801 ACGGAGAGGTCATCCGCTCGACGGCGCATTCGCGATGGCCCCCAAGTGAAGGAGCGTTT
-----+----- 1860
TGCCTCTCCAGTAGGCGGAGCTGCCGCGTAACGCGTACCGGGGGTTCACTTCTCGCAAA

1861 CATGGACCCCATCGTCATCACGGGCGCGATGCGCACCCCGATGGGGGCATTCCAGGGCGA
-----+----- 1920
GTACCTGGGGTAGCAGTAGTGGCGCGCTACGCGTGGGGCTACCCCGTAAGGTCCCGCT

1921 TCTTGGCCCGATGGATGCCCGGACCTTGGCGCGGACGCGATCCGCGCGCGCTGAACGG
-----+----- 1980
AGAACGGCGCTACCTACGGGCTGGGAACCGCGCCTGCCTAGGCGCGCGCGCACTTGCC

```

Fig. 24/3

```

1981 CCTGTCGCCCCGACATGGTGGACGAGGTGCTGATGGGCTGCGTCCTCGCCGCGGGCCAGGG
-----+-----+-----+-----+-----+-----+-----+-----+-----+
2040 GGACAGCGGGCTGTACCACCTGCTCCACGACTACCCGACGCGAGGAGCGGCGCCCGGTCCC
-----+-----+-----+-----+-----+-----+-----+-----+-----+

2041 TCAGGCACCGGCACGTCAAGCGGGCGCTTGGCGCCGACTGCCGCTGTGACGGGCACGAC
-----+-----+-----+-----+-----+-----+-----+-----+-----+
2100 AGTCCGTGGCCGTGCAGTCCGCCGCGAACC CGCGGCTGACGGCGACAGCTGCCCGTGCTG
-----+-----+-----+-----+-----+-----+-----+-----+-----+

2101 CACCATCAACGAGATGTGCGGATCGGGCATGAAGGCCGCGATGCTGGGCCATGACCTGAT
-----+-----+-----+-----+-----+-----+-----+-----+-----+
2160 GTGGTAGTTGCTCTACACGCTAGCCCGTACTTCCGCGCTACGACCCGGTACTGGACTA
-----+-----+-----+-----+-----+-----+-----+-----+-----+

2161 CGCCGCGGGATCGGCGGGCATCGTCTGCGCGGGGGATGGAGAGCATGTGGAACGCCCC
-----+-----+-----+-----+-----+-----+-----+-----+-----+
2220 GCGGCGCCCTAGCCGCCCGTAGCAGCAGCGGCGGCCCTACCTCTCGTACAGCTTGGGGG
-----+-----+-----+-----+-----+-----+-----+-----+-----+

2221 CTACCTGCTGCCAAGGCGCGGTGCGGGATGCGCATGGGCCATGACCGTGTGCTGGATCA
-----+-----+-----+-----+-----+-----+-----+-----+-----+
2280 GATGGACGACGGGTTCCGCGCCAGCCCCCTACGCGTACCCGGTACTGGCACACGACCTAGT
-----+-----+-----+-----+-----+-----+-----+-----+-----+

2281 CATGTTCTCTGACGGGTTGGAGGACGCCATATGACAAGGCGCGCTGATGGGCACCTTCGC
-----+-----+-----+-----+-----+-----+-----+-----+-----+
2340 GTACAAGGAGCTGCCAACCTCTGCGGATACTGTTCCCGCGGACTACCCGTGGAAGCG
-----+-----+-----+-----+-----+-----+-----+-----+-----+

2341 CGAGGATTGCGCCGCGGATCAGCGTTTCACCCGCGAGGCGCAGGACGACTATGCGCTGAC
-----+-----+-----+-----+-----+-----+-----+-----+-----+
2400 GCTCCTAACGCGGCCGCTAGTGCCAAAGTGGGCGCTCCGCGTCTGCTGATACGCGACTG
-----+-----+-----+-----+-----+-----+-----+-----+-----+

2401 CAGCCTGGCCCGCGCGCAGGACGCCATCGCCAGCGGTGCCCTTCGCCCGCGAGATCGCGCC
-----+-----+-----+-----+-----+-----+-----+-----+-----+
2460 GTGGGACCGGGCGCGCTCTGCGGTAGCGGTGCGCCACGGAAGCGGCGGCTCTAGCGCGG
-----+-----+-----+-----+-----+-----+-----+-----+-----+

2461 CGTGACCGTCACGGCAGCAAGGTGCAGACCACCGTCGATACCGACGAGATGCCCGGCAA
-----+-----+-----+-----+-----+-----+-----+-----+-----+
2520 GCACTGGCAGTGCCGTGCGTTCCACGTCTGGTGGCAGCTATGGCTGCTCTACGGGCGGTT
-----+-----+-----+-----+-----+-----+-----+-----+-----+

2521 GGCCCGCCCCGAGAAGATCCCCCATCTGAAGCCCGCCTTCCGTGACGGTGGCAGGTCAC
-----+-----+-----+-----+-----+-----+-----+-----+-----+
2580 CCGGGCGGGGCTCTTCTAGGGGTAGACTTCGGGCGGAAGGCACTGCCACCGTGCCAGTG
-----+-----+-----+-----+-----+-----+-----+-----+-----+

2581 GGCGGCGAACAGCTCGTCGATCTCGGACGGGGCGCGGGCGCTGGTGATGATGCCCCAGTC
-----+-----+-----+-----+-----+-----+-----+-----+-----+
2640 CCGCGGCTTGTGAGCAGCTAGAGCCTGCCCCGCGCGCGGACCACTACTACGGGCTCAG
-----+-----+-----+-----+-----+-----+-----+-----+-----+

2641 GCAGGCGGAGAAGCTGGGCCTGACGCCGATCGCGCGGATCATCGGTGATGCGACCCATGC
-----+-----+-----+-----+-----+-----+-----+-----+-----+
2700 CGTCCGGCTCTTCGACCCGGACTGCGGCTAGCGCGCCTAGTAGCCAGTACGCTGGGTACG
-----+-----+-----+-----+-----+-----+-----+-----+-----+

2701 CGACCGTCCCGGCTGTTCCCGACGSCCCCATCGGCGCGATGCGCAAGCTGCTGGACCG
-----+-----+-----+-----+-----+-----+-----+-----+-----+
2760 GCTGGCAGGGCCGGACAAGGGCTGCCGGGGTAGCCGCGCTACGCGTTCGACGACCTGGC
-----+-----+-----+-----+-----+-----+-----+-----+-----+

2761 CACGGACACCCGCTTGGCGATTACGACCTGTTGAGGTGAACGAGGCATTGCGCGTCGT
-----+-----+-----+-----+-----+-----+-----+-----+-----+
2820 GTGCCTGTGGGCGGAACCGCTAATGCTGGACAAGCTCCACTTGCTCCGTAAGCGGCAGCA
-----+-----+-----+-----+-----+-----+-----+-----+-----+

2821 CGCCATGATCGCGATGAAGGAGCTTGGCCTGCCACACGATGCCACGAACATCAACGCGG
-----+-----+-----+-----+-----+-----+-----+-----+-----+
2880 GCGGTACTAGCGCTACTTCTCGAACCGGACGGTGTGCTACGGTGCTTGTAGTTGCCGCC
-----+-----+-----+-----+-----+-----+-----+-----+-----+

2881 GGCCTCGCGGCTTGGSCATCCCATCGCGCGCTCGGGGGCGCGGATCATGGTCACGCTGCT
-----+-----+-----+-----+-----+-----+-----+-----+-----+
2940 CCGGACCGCGGAACCCGTAGGCTAGCCCGCAGCCCCCGCGCTAGTACCAGTGGCAGCA
-----+-----+-----+-----+-----+-----+-----+-----+-----+

2941 GAACGCGATGCGGGCGCGGGGCGGACGCGCGCGGCGCGCATCCGTCTGCA TCGGCGGGGG
-----+-----+-----+-----+-----+-----+-----+-----+-----+
3000 CTTGCGCTACCGCGCGCCCGCGCTGCGCGCCCCCGCGGTAGGCAGACGTAGCCGCCCCC
-----+-----+-----+-----+-----+-----+-----+-----+-----+

```

87

Fig. 24/5

GTTCCGCGGGGGCAGATCGCGGACCTGCTGGCCGCGTGCTGCCGATGACATCCGCGG  
 4021 ----- 4080  
 CAAGGCGGCCCCCTCTAGCGCCTGGACGACCGGGCGCAGACGCGCTACTGTAGGCGGC  
 CAGCGCCTAGGCGCGGGTCCGGTCCACAGGCGGTCGCGGCTGATTTGCCGCGCGCGCAG  
 4081 ----- 4140  
 GTCGCGGATCCGCGCGCCAGCCAGGTGTCCGCGAGCGCCGACTAAAGCGGCGGCGCGTC  
 GCGGATGCGGCGCGCTCCAAGCCTCCGCGCGCCAGAAGCCCGATCTTGGCAGCCTTCGA  
 4141 ----- 4200  
 CGCGCTACGCGCGCGCAGGTTCGGAGCGCGCGGTCTTCGGGCTAGAACCCTCGGAAGCT  
 CGTGCTGATCCGCTGGCGATAGGCCTCGGGGCCACCTGCCGATGCGCGTCCCGATTGC  
 4201 ----- 4260  
 GCACGACTAGCGGACCGCTATCCGGAGCCCCGGTGGGACGGCCTACGCGCAGGGCTAACG  
 GCGATAGATACGCGCGCGCGCGGATCGACCACGCGCAGCGCGCGCGCAGATGCGGGAAG  
 4261 ----- 4320  
 CGCTATCTATGCGTCCGCGCGCGCTAGCTGGTGGCGGTCCGCGCGCGCTTACGCGCTTC  
 CCCCTGCCGCGCGGAGGCATAATAGGGCTCGGCGCGCTCAAGCAGGCGGATGATGACGGA  
 4321 ----- 4380  
 GGGGACGGCGCGGCTCCGTATTATCCCGAGCGCGCGCAGTTCTGCCGCTACTACTGCTT  
 ATAGAGCGCTCCGAAGGCACCGGACCTCAACCGTCCCCCGCGCTCGGCCAGCCAGTC  
 4381 ----- 4440  
 TATCTCGCGCAGGCTTCCGTGGCTGGGAGTTGGCAGCGGGGGCGGAGCCGGTCGGTCAG  
 GGCAGGCAGATAGCAGCGCCCGATGGCGGCATCGTCGATCACGTCCGAGCGATGTTCCGT  
 4441 ----- 4500  
 CCGTCCGTCTATCGTCGCGGGCTACCGCGGTAGCAGCTAGTGCGAGCGCTCGCTACAAGCA  
 CAGCTGGAACGCAAGGCCAGATCGCAGGCGGSAATCCAGCACCAGCATCGTCTGACGCGC  
 4501 ----- 4560  
 GTCGACCTTGGCTTCCGGTCTAGCGTCCGCGCTAGGTCTGGCGTAGCAGGACGTGCGG  
 CATCACCGCGCCATCATCACGCCACGACCGCGCGAGCTGGTAGGAATATTCCAGCAC  
 4561 ----- 4620  
 GTAGTGGGCGCGGTAGTAGTGCGGGTGTGGGGCGCTGCACCATCCTTATAAGGTCGTG  
 GTCATCCAGGCTGCGGTATTCCGATCCGCGACATCCATCGCGAAACCTCGATCAGGTC  
 4621 ----- 4680  
 CAGTAGGTCCGACGCCATAAGCGCTAGCGCTGTAGGTAGCGCTTGGGAGCTAGTCCAG  
 CATCGGCCAAAGGTCCGGGAAATCATGCCGCGGGCGACCTGGCGCAGCGCGCGAAGGG  
 4681 ----- 4740  
 GTAGCCGGTTTCCAGGCCCTTTAGTACGGCGGCGCGCTGGACCGCGTCGCGGCGCTTCCC  
 CGCGGACATCGGGCCCTCCTCGTGACGCGCGCCAGCGTGTGGCGCGCAGCGCCCCCAG  
 4741 ----- 4800  
 GCGGCTGTAGCCCGGAGGAGCACCTCGCGCGGTGCGACAGCCGCGCTCGCGGGGGTC  
 CGCGCGCTGTGGTCCGCGCGCGCTCGGGGCGAGAACCACCTACCTGCCCGTCGATCAC  
 4801 ----- 4860  
 GGCGCGGACACCCAGCGCGGGCGAGCCCCCGTCTGGGTAGTGGACGGGCGAGCTAGTG  
 GTCATCCGCAATGCTGACAGGCGATAGAGCATGACCGTATCCTCGCGGATGCGGGGGGG  
 4861 ----- 4920  
 CAGTAGGCGTACGGACGTGGTCCGTATCTCGTACTGGCATAGGAGCGCTACGGCCCCGC  
 CATCAGCTTGGCGCGCTGGCGGAAGCTTTGCGAACCCTCGCGGATGGCGCGCTTCGGAAGT  
 4921 ----- 4980  
 GTAGTCGAACCGGCGGACGCGCTTCGAAACGCTTGGGACCGGCTACCGGCGAAGCCTTCA  
 CGCGCTCAGATCGGTATCGGACGGCCAGGTCCGACAGCATGACCTGCGCGGTGGCCTTG  
 4981 ----- 5040  
 GCGGCAGTCTAGCCAGTACCGTCCCGGTCCAGGCTGTGCTACTGGACGGCGCACCGGAAC

89

BNSDOCID: <EP\_\_\_\_0872554A2\_I\_>

Fig. 24/8

7021 CGCCGTCGCTGTAGCCGCTATCCTCGATCAGGATGCGGGTGGGACTGAAGGGCAGCAGAT  
 -----+----- 7080  
 GCGGCAGCGACATCGCCCATAGGAGCTAGTCCTACGCCACCGTGACTTCCCGTCGTCTA  
 7081 AGATGAAGCGGTACCCGTCATCTGCGGAACGGTCGGTCCATGATCATCGGGCGCTCGA  
 -----+----- 7140  
 TCTACTTCGCCATGGGCAGGTAGACGGCTTCCAGCGCAGGTACTAGTAGCCCGCGAGCT  
 7141 CGCCATGGGGGGCGTCCGTCTCGATCTGACGCCACGAATTTCTGGAACCCACGGTCA  
 -----+----- 7200  
 GCGGTACCCCCCGCAGCCAGAGCTAGAGCTGCGGGTGCTTAAAGACCTTTGGGTGCCAGT  
 7201 GGTGCGGGGTCTCGACGGCACCACGGGCGTCSATCAGCAGGCAGCCTCGATCCGCGAGC  
 -----+----- 7260  
 CCACGCCCCAGAGTGCCGTGGTGGCCGAGCTAGTGCGTCCGTCCGAGCTAGGCGCTCG  
 7261 CGTCCGTCAGCGTCCGGCCGCTATCGTCCAGCGTCGCGACATGCGTATTCCACCGCAGAT  
 -----+----- 7320  
 GCAGGCAGTCGCAGCGCGGCCATAGCAGGTCCGACGCGCTGTACGCATAAGGTGGCGTCTA  
 7321 CGACACCCTGCAGCAGCCCGATCAGCGCGCCCGCCTCGATCGAGCCATAGCCTGTCTGCA  
 -----+----- 7380  
 GCTGTGGGACGTCTCGGGCTAGTCCCGGGCGGAGCTAGCTCGGTATCGGACAGCAGT  
 7381 GCGGGCGGAATGGTCCGGAAACGGCAGCCTCCTGATCCGTCCATTGCGCGCGACGAATGG  
 -----+----- 7440  
 CCGCGCGCTTACCAGCCCTTTGCGCTGGAGGACTAGGCAGGTAAGCGGCGCTGCTTACC  
 7441 GCGACAGGCGCGCCAGCCATTGCGGCGAAAGATCCGTGTCTGTGGCAGGACCAGGTGTGCT  
 -----+----- 7500  
 CGCTGTCCGCGCGGTCCGTAAGCCCGCTTTCTAGGCACAGCACCGTCTTGGTCCACACGA  
 7501 GGTCCGAGGGGCCGGACCGCGCGTCCAGCATCACGATGCGCGCATCCGGTCTGCGGTCCG  
 -----+----- 7560  
 CCAGGCTCCCCGGCCTGGCGCGCAGCTCGTAGTGCTACGCGCGTAGGCCAGACGCCAGCG  
 7561 GAACGGCAAGCGCGATCAGCGCACCGGACAGCCCCGCGCCCGCATCAGCAGATCATGGC  
 -----+----- 7620  
 CTTGCCGTTCCGCGCTAGTCCGCTGGCCTGTGCGGGCGCGGGCGCTAGTCTCTAGTACCG  
 7621 TCATGTATTGCGATCCGCCCTTCCCGGTCTTCAGCAGCGCGCCCGAGCGTTTCAGCTC  
 -----+----- 7680  
 AGTACATAACGCTAGGCGGGGAAGCGCCAGGAAGTCGTGCGCGCGGCTCGCAAAGTCGAG  
 7681 TGCCTTGAGGCTGTGACCGAGGGCGGCCAGATGAAACCGAAGCTGACGCAGTTCTCGCG  
 -----+----- 7740  
 ACGGAACCTCCGACAGCTGGCTCCCGCGGGTCTACTTTGGCTTCGACTGCGTCAAGAGCGC  
 7741 GCCATGGACCGCGTGTATGATCTGTGTGCTGGTAGACCGGACGAAGATAGCCGCGCTT  
 -----+----- 7800  
 CCGGTACCTGGCGCACTACGTAGGACACACGGACCATCTGCGCTGCTTCTATCGGCGCGAA  
 7801 GGGGACATAGCGGAACGGCCAGCCCGCATGCCAACCGGTCTATGCAGGAAATAGTAGAT  
 -----+----- 7860  
 CCGCTGTATCCGCTTCCCGGTCCCGGGTACGTGGTTCCGCGTACGTCTTTATCATCTA  
 7861 CAGCCCGTAGCAGGTGACCCCGACCGCCAGCCACCAAGCCAGATCCGACCCCATCGCGCC  
 -----+----- 7920  
 GTCGGGCATCGTCCACTGGGGGTGGCGGTGGTGGTCCGGTCTAGGCTGGGGTAGCGCGG  
 7921 GATCGCGAACAGCAGATCGAGATTACCGCGAAGATGACGCCATAGAGGTGCTTCTCTC  
 -----+----- 7980  
 CTAGCGCTTGTGCTGCTAGCTCTAATGCGCGTCTACTGCGGTATCTCCAGCAAGAAGAG

92

Fig. 24/10

ATACCGTCGACCTCGAGGGGGGGGGGGTACCCAGCTTTTGTTCCTTTAGTGAGGGTTA  
 9001 -----+----- 9060  
 TATGGCAGCTGGAGCTCCCCCGGGCCATGGGTGAAAACAGGGCAAATCACTCCCAAT  
 ATGCGCGCTTGGCCTAATCATGGTCATAGCTTTTCTGTGTGAAATTGTTATCCGCTC  
 9061 -----+----- 9120  
 TAACGGCGGAACCGCATTAGTACCAGTATCGACAAAGGACACACTTTAACAATAGGCGAG  
 ACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAGCCTGGGGTGCCTAATGA  
 9121 -----+----- 9180  
 TGTTAAGGTGTGTGTATGCTCGGCCTTCGTATTTCAATTTTCGGACCCACGGATTACT  
 GTGAGCTAACTCAGTTAATTGCGTTGCGCTCACTGCCCCGCTTTCCAGTCGGGAAACCTG  
 9181 -----+----- 9240  
 CACTCGATTGAGTGAATTAACGCAACGCGAGTGACGGGCGAAAGGTCAGCCCTTTGGAC  
 TCGTGCCAGCTGCATTAAATGAATCGGCCAACCGCGGGGAGAGGCGGTTTGCGTATTGGG  
 9241 -----+----- 9300  
 AGCACGGTCGACGTAATTACTTAGCCGGTTGCGGCCCCCTCTCCGCCAAACGCATAACCC  
 CGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCTGTTCCGGCTGCGGCGAGCG  
 9301 -----+----- 9360  
 GCGAGAAGGCGAAGGAGCGAGTGACTGAGCGACGCGAGCCAGCAAGCCGACGCGCTCGC  
 GTATCAGCTCACTCAAAGGCGGTAAATACGGTTATCCACAGAATCAGGGGATAACGCAGGA  
 9361 -----+----- 9420  
 CATAGTCGAGTGAGTTTCCGCCATTATGCCAATAGGTGTCTTAGTCCCTATTGCGTCCCT  
 AAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTG  
 9421 -----+----- 9480  
 TTCTTGTAACACTCGTTTCCGGTGGTTTCCGGTCCCTTGGCATTTTTCCGGCGCAACGAC  
 GCGTTTTTCCATAGGCTCCGCCGCCCTGAGGAGCATCACAAAAATCGACGCTCAAGTCAG  
 9481 -----+----- 9540  
 CGCAAAAGGTATCCGAGGCGGGGGGACTGCTCGTAGTGTTTTTAGCTGCGAGTTCACTC  
 AGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTC  
 9541 -----+----- 9600  
 TCCACCGCTTTGGGCTGTCCTGATATTTCTATGGTCCGCAAGGGGGACCTTCGAGGGAG  
 GTGCGCTCTCCTGTTCGACCCTCGCGCTTACCGGATACCTGTCCGCTTTCTCCCTTCG  
 9601 -----+----- 9660  
 CACGCGAGAGGACAAGGCTCGGACGGCGAATGGCTATGGACAGGCGGAAAGAGGGAAGC  
 GGAAGCGTGGCGCTTTCTCATAGCTCAGCTGTAGGTATCTCAGTTCGGTGTAAGTCTGT  
 9661 -----+----- 9720  
 CCTTCGCACCGCGAAAGAGTATCGAGTGGGACATCCATAGAGTCAAGCCACATCCAGCAA  
 CGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTTCAGCCCGACCGCTGCGCCTTATCC  
 9721 -----+----- 9780  
 GCGAGGTTTCGACCCGACACACGCTTGGGGGGCAAGTCGGGCTGGCGACGCGGAATAGG  
 GGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCC  
 9781 -----+----- 9840  
 CCATTGATAGCAGAACTCAGGTTGGGCCATTCTGTGCTGAATAGCGGTGACCGTCTGTCGG  
 ACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGG  
 9841 -----+----- 9900  
 TGACCAATTGTCCTAATCGTCTCGCTCCATACATCCGCCACGATGTCTCAAGAACTTCACC  
 TGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCA  
 9901 -----+----- 9960  
 ACCGGATTGATGCCGATGTGATCTTCTGTCTATAAACCATAGACGCGAGACGACTTCGGT

BNSDOCID: <EP\_0872554A2 | >

Fig. 24/12

```

10981  ACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCA
-----+----- 11040
      TGAGCACGTGGGTTGACTAGAACTCGTAGAAAATGAAAAGTGGTCGCAAGACCCACTCGT
      AAAACAGGAAGGCCAAAATGCCGCAAAAAAGGGAAATAGGGCGACACGGAAATGTTGAATA
11041  -----+----- 11100
      TTTTGTCTTCCGTTTACGGCGTTTTTTCCCTTATTCGCGCTGTGCCTTTACAACTTAT
      CTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGC
11101  -----+----- 11160
      GAGTATGAGAAGGAAAAAGTTATAATAACTTCGTAAATAGTCCCAATAACAGAGTACTCG
      GGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCC
11161  -----+----- 11220
      CCTATGTATAAACTTACATAAAATCTTTTTATTTGTTTATCCCCAAGGCGCGTGTAAAGGG
      CGAAAAGTGCCAC
11221  -----+----- 11233
      GCTTTTCACGGTG

```

BNSDOCID: <EP\_\_\_\_0872554A2\_1\_>

[illegible]

Fig. 26

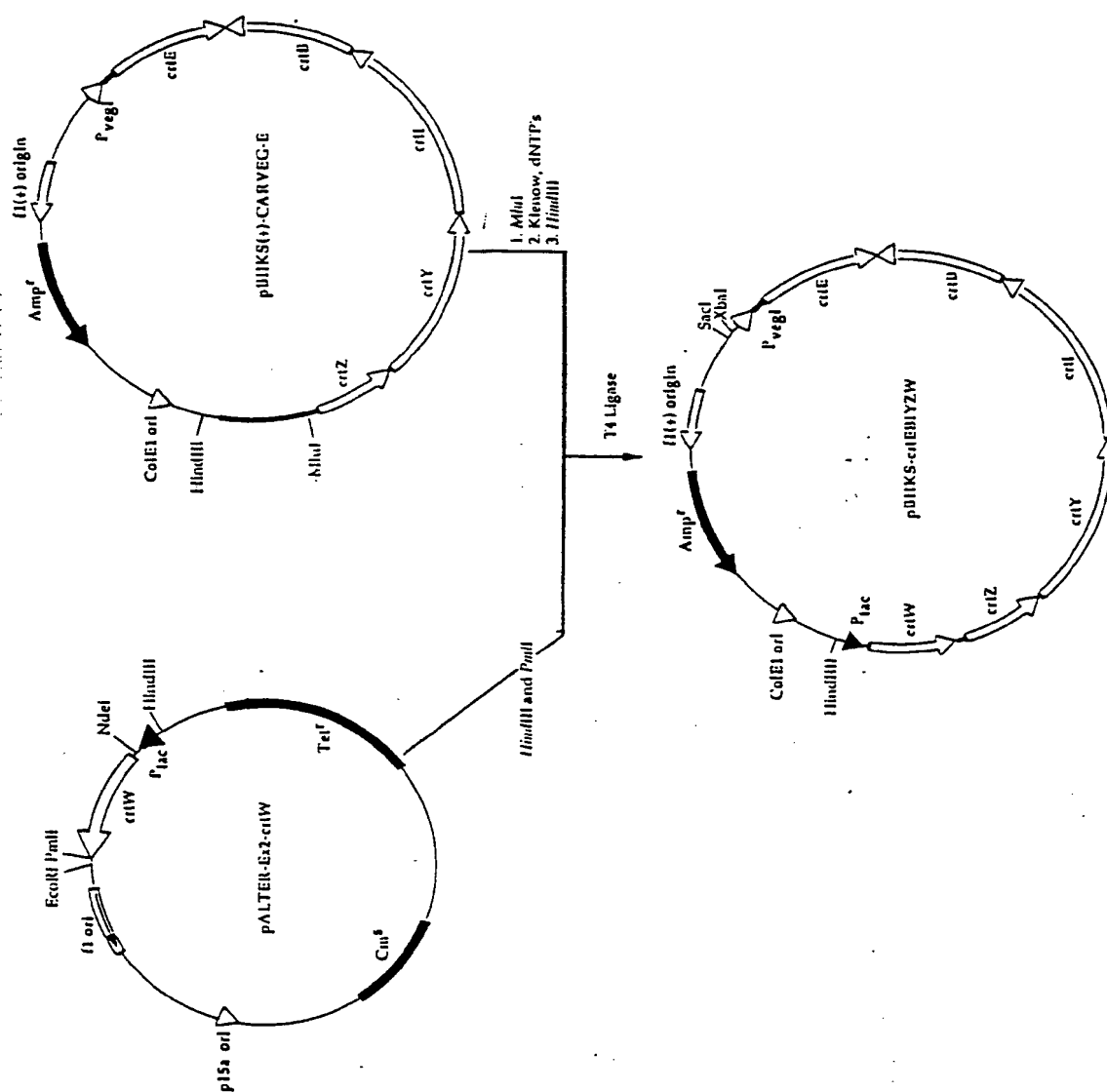


Fig. 27

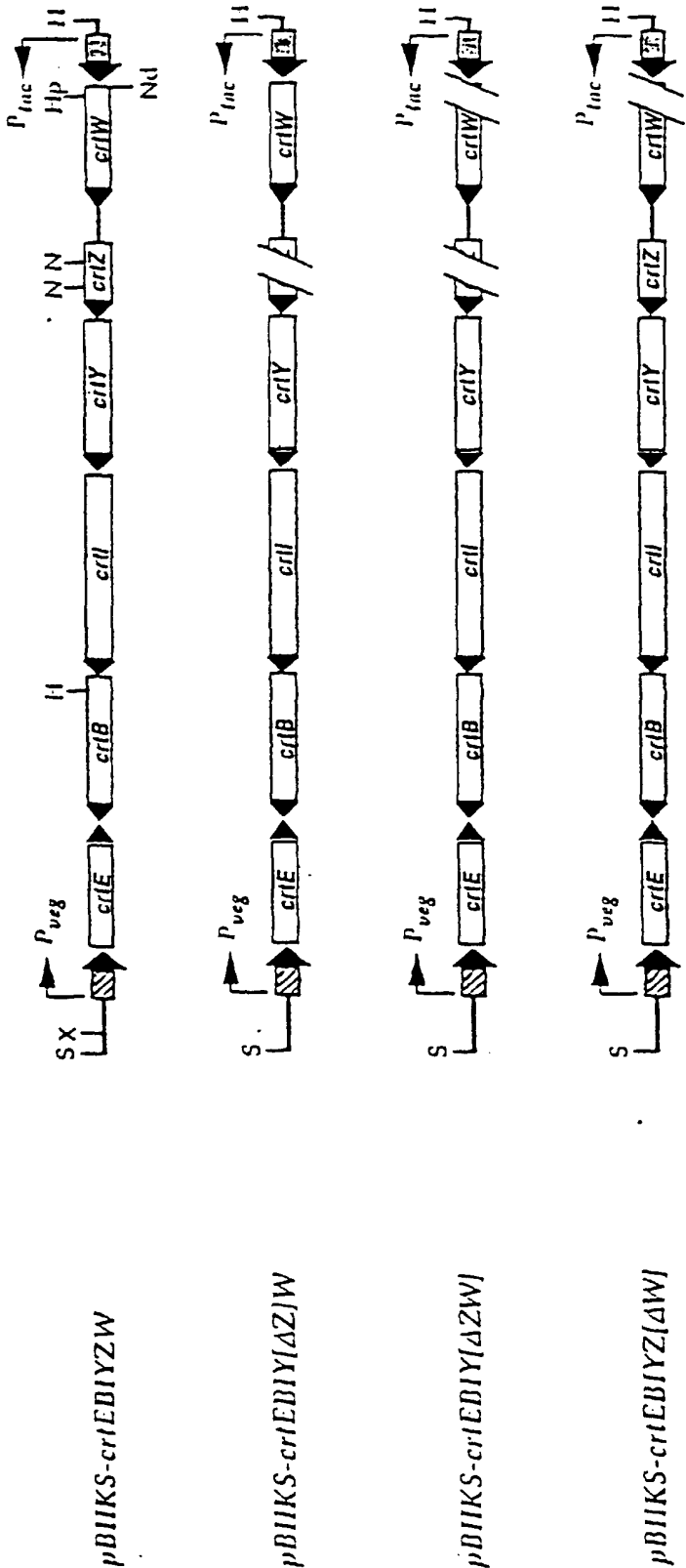


Fig. 28

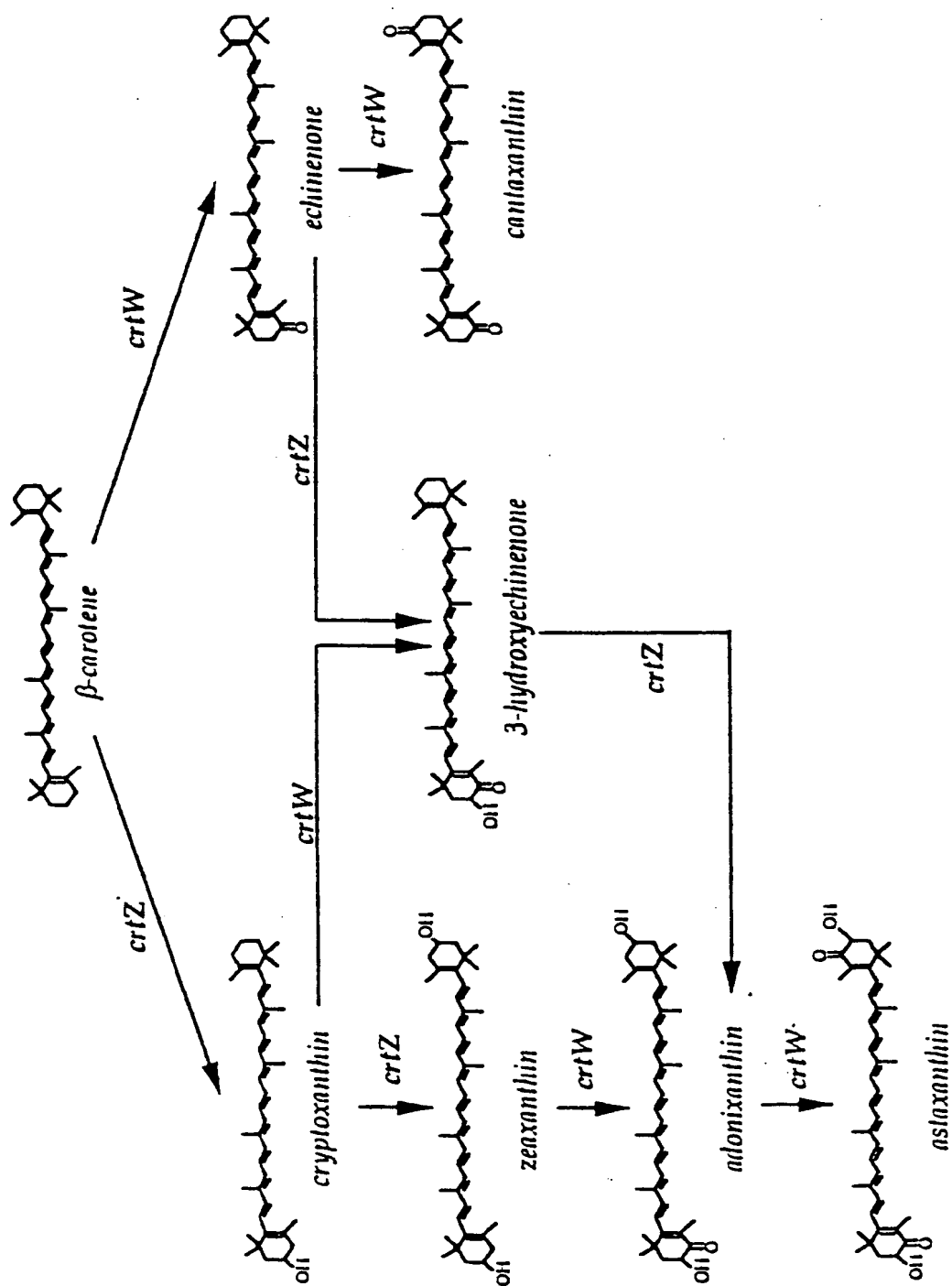


Fig. 29

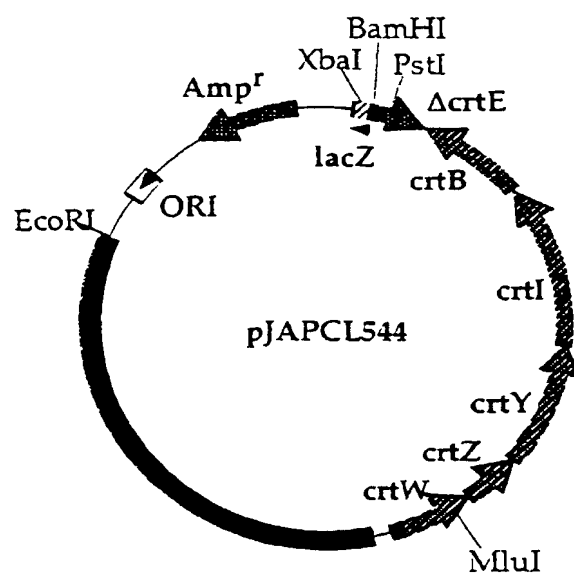


Fig. 30/1

```

1  ACTGTAGTCTGCGCGGATCGCCGGTCCGGGGGACAAGATATGAGCGCACATGCCCTGCC
   -----+-----+-----+-----+-----+-----+
61  TGACATCAGACGCGCCTAGCGGCCAGGCCCTGTTCTATACTCGCGGTACGGGACGGG
   -----+-----+-----+-----+-----+-----+
   AAGGCAGATCTGACCGCCACCAGTTTGATCGTCTCGGGGGGCATCATGCCCGGTGGCTG
61  -----+-----+-----+-----+-----+-----+
   TTCCGTCTAGACTGGCGGTGGTCAAAGTAGCAGAGCCCGCGTAGTAGCGGGCGACCGAC
   -----+-----+-----+-----+-----+-----+
121  GCCCTGCATGTGCATGCGCTGTGGTTTCTGGACGCGCGGGCGCATCCCATCCTGGCGGTC
   -----+-----+-----+-----+-----+-----+
121  CGGGACGTACACGTACCGGACACCAAAGACCTGCGCGCGCGCGTAGGGTAGGACCGCCAG
   -----+-----+-----+-----+-----+-----+
   GCGAATTTCTGGGGCTGACCTGGCTGTGCGGTGCGTCTGTTCATCATCGCGCATGACGGG
181  -----+-----+-----+-----+-----+-----+
   CGCTTAAAGGACCCGACTGGACCGACAGCCAGCCAGACAAGTAGTAGCGCGTACTGCGC
   -----+-----+-----+-----+-----+-----+
241  ATGCATGGGTGCGTGTGCGGGGGCGCCGCGCGCAATGCGGCGATGGGCCAGCTTGTC
   -----+-----+-----+-----+-----+-----+
241  TACGTACCCAGCCAGCACGCGCCCGCGGGCGCGCGTTACGCCGCTACCCGGTCGAACAG
   -----+-----+-----+-----+-----+-----+
   CTGTGGCTGTATGCCGGATTTTCTGGCGCAAGATGATCGTCAAGCACATGGCCCATCAT
301  -----+-----+-----+-----+-----+-----+
   GACACCGACATACGGCCTAAAAGGACCGCGTTCTACTAGCAGTTCGTGTACCGGGTAGTA
   -----+-----+-----+-----+-----+-----+
   CGCCATGCGGGAACCGACGACGACCCAGATTTGACCATGGCGGCGCGGTCCGCTGGTAC
361  -----+-----+-----+-----+-----+-----+
   GCGGTACGGCCTTGCTGTGCTGGGTCTAAAGCTGGTACCGCGGGCCAGGCGACCATG
   -----+-----+-----+-----+-----+-----+
   GCCCGCTTCATCGGCACCTATTTGCGGTGGCGGAGGGGCTGCTGCTGCCCGTCACTGTG
421  -----+-----+-----+-----+-----+-----+
   CGGGCGAAGTAGCCGTGGATAAAGCCGACCGCGCTCCCGACGACGACCGGCAGTAGCAC
   -----+-----+-----+-----+-----+-----+
   ACGGTCTATGCGCTGATGTTGGGGGATCGCTGGATGTAAGTGGTCTTCTGGCCGTGCGG
481  -----+-----+-----+-----+-----+-----+
   TGCCAGATACGCGACTACAACCCCTAGCGACCTACATGCACCAGAAGACCGGCAACGGC
   -----+-----+-----+-----+-----+-----+
   TCGATCCTGGCGTCGATCCAGCTGTTCTGTGTTCCGGCATCTGGCTGCCGACCGCCCGGC
541  -----+-----+-----+-----+-----+-----+
   AGCTAGGACCGCAGCTAGGTGACAAGCACAAAGCCGTAGACCGGACGGCGTGGCGGGCCG
   -----+-----+-----+-----+-----+-----+
   CACGACGCGTTCGCGGACCGCCACAATGCGCGGTGCTGCGGGATCAGCGACCCCGTGTG
601  -----+-----+-----+-----+-----+-----+
   GTGCTGCGCAAGGGCCTGGCGGTGTACGCGCCAGCAGCGCCTAGTCTGCTGGGGCACAGC
   -----+-----+-----+-----+-----+-----+
   CTGCTGACCTGCTTTCACTTTGGCGGTTATCATCACGAACACCCTGCACCCGACGGTG
661  -----+-----+-----+-----+-----+-----+
   GACGACTGGACGAAAGTGAACCCGCAATAGTAGTGCTTGTGGTGGACGTGGGCTGCCAC
   -----+-----+-----+-----+-----+-----+
   CCTTGGTGGCGCCTGCCCAGCACCCGACCAAGGGGGACACCGCATGACCAATTTCTCTGA
721  -----+-----+-----+-----+-----+-----+
   GGAACCAACCGGACGGGTGCTGGGCGTGGTTCCCCCTGTGGCGTACTGGTTAAAGGACT
   -----+-----+-----+-----+-----+-----+
   TCGTCTGCGCACCGTGTGTTGATGGAGCTGACGGCCTATTCGCTCCACCGCTGGATCA
781  -----+-----+-----+-----+-----+-----+
   AGCAGCAGCGGTGGCACGACCACTACCTCGACTGCCGATAAGGCAGGTGGCGACCTAGT
   -----+-----+-----+-----+-----+-----+

```

Fig. 30/2

```

841 TGCACGGCCCCCTTGGGCTGGGGCTGGCACAAGTCCCACCACGAGGAACACGACCACGGCGC
-----+-----+-----+-----+-----+-----+-----+
ACGTGCCGGGGAAACCCGACCCGACCGTGTTTCAGGGTGGTGCTCCTTGTGCTGGTGCCGG
900

TGGAAAAGAACGACCTGTACGGCCTGGTCTTTGCGGTGATCGCCACGGTGCTGTTTCACGG
-----+-----+-----+-----+-----+-----+-----+
901 ACCTTTTCTTGCTGGACATGCCGGACCAGAAACGCCACTAGCGGTGCCACGACAAGTGCC
960

TGGGCTGGATCTGGGCACCGGTCCTGTGGTGGATCGCCTTGGGCATGACCGTCTACGGGC
-----+-----+-----+-----+-----+-----+-----+
961 ACCCGACCTAGACCCGTGGCCAGGACACCACCTAGCGGAACCCGTACTGGCAGATGCCCG
1020

TGATCTATTTCGTCTGCATGACGGGCTGGTGCATCAGCGCTGGCCGTTCCGCTATATCC
-----+-----+-----+-----+-----+-----+-----+
1021 ACTAGATAAAGCAGGACGTACTGCCCGACCACGTAGTCGCGACCGGCAAGGCGATATAG
1080

CTCGCAAGGGCTATGCCAGACGCCTGTATCAGGCCCACCGCCTGCACCACGGGTCGAGG
-----+-----+-----+-----+-----+-----+-----+
1081 GAGCGTTCCCGATACGGTCTGCGGACATAGTCCGGGTGGCGGACGTGGTGCGCCAGCTCC
1140

GGCGCGACCATTGCCTCAGCTTCGGCTTCATCTATGCGCCGCCGGTCGACAAGCTGAAGC
-----+-----+-----+-----+-----+-----+-----+
1141 CCGCGCTGGTAACGCAGTCGAAGCCGAAGTAGATACGCGGCGGCCAGCTGTTGACTTCG
1200

AGGACCTGAAGACGTGGGGCGTGCTGCGGGCCGAGGCGCAGGAGCGCACGTGACCCATGA
-----+-----+-----+-----+-----+-----+-----+
1201 TCCTGGACTTCTGCAGCCCGCACGACGCCCGGGCTCCGCGTCTCGCGTGCACTGGGTACT
1260

C
1261 - 1261
G

```

Fig. 31

```

1  ATGAGCGCACATGCCCTGCCCAAGGCAGATCTGACCGCCACCAGTTTGATCGTCTCGGGC
   -----+-----+-----+-----+-----+-----+ 60
   TACTCGCGTGACGGGACGGGTTCGGTCTAGACTGGCGGTGGTCAAACCTAGCAGAGCCCG

61  GGCATCATCGCCGCGTGGCTGGCCCTGCATGTGCATGCGCTGTGGTTTCTGGACGCGGCG
   -----+-----+-----+-----+-----+-----+ 120
   CCGTAGTAGCGGCGCACCGACCGGGACGTACACGTACGCGACACCAAAGACCTGCGCCGC

121  GCGCATCCCATCCTGGCGGTGCGGAATTTCTGGGGCTGACCTGGCTGTGGTTCGGTCTG
   -----+-----+-----+-----+-----+-----+ 180
   CGCGTAGGGTAGGACCGCCAGCGCTTAAAGGACCCGACTGGACCGACAGCCAGCCAGAC

181  TTCATCATCGCGCATGACGCGATGCATGGGTGCGTCTGCGGGGGCGCCCGCGGCCAAT
   -----+-----+-----+-----+-----+-----+ 240
   AAGTAGTAGCGGTACTGCGCTACGTACCCAGCCAGCACGGCCCCGCGGCGCGGTTA

241  GCGGCGATGGGCCAGCTTGCTCTGTGGCTGTATGCCGATTTTCTGGCGCAAGATGATC
   -----+-----+-----+-----+-----+-----+ 300
   CGCGCGCTACCGGTGGAACAGGACACCGACATACGGCCTAAAAGGACCGCGTTCTACTAG

301  GTCAAGCACATGGCCCATCATCGCCATGCCGGAACCGACGACGCCAGATTTCGACCAT
   -----+-----+-----+-----+-----+-----+ 360
   CAGTTCTGTGTACCGGTAGTAGCGGTACGGCCTTGGCTGCTGCTGGGTCTAAAGCTGGTA

361  GGCGGCCCGGTCCCGTGGTACGCCCGCTTCATCGGCACCTATTTCGGCTGGCGCGAGGGG
   -----+-----+-----+-----+-----+-----+ 420
   CCGCGGGGCGAGCGACCATGCGGGCGAAGTAGCCGTGGATAAAGCCGACCGCGCTCCCC

421  CTGCTGCTGCCCGTCATCGTGACGGTCTATGCGCTGATGTTGGGGATCGCTGGATGTAC
   -----+-----+-----+-----+-----+-----+ 480
   GACGACGACGGGCAGTAGCACTGCCAGATACGCGACTACAACCCCTAGCGACCTACATG

481  GTGGTCTTCTGGCCGTTCGGTTCGATCCTGGCGTCGATCCAGCTGTTCTGTGTTCCGGCATC
   -----+-----+-----+-----+-----+-----+ 540
   CACCAGAAGACCGGCAACGGCAGCTAGGACCGCAGCTAGGTCGACAAGCACAGCCGTAG

541  TGGCTGCGGCACCGCCCGGGCCACGACGCGTTCCCGGACCGCCACAATGCGCGGTCTGTCG
   -----+-----+-----+-----+-----+-----+ 600
   ACCGACGGCGTGGCGGGGCGGTGCTGCGCAAGGGCTGGCGGTGTTACGCGCCAGCAGC

601  CGGATCAGCGACCCCGTGTGCTGCTGACCTGCTTTCACTTTGGCGGTTATCATCACGAA
   -----+-----+-----+-----+-----+-----+ 660
   GCCTAGTCTGCTGGGGCACAGCGACGACTGGACGAAAGTGAAACCGCCAATAGTAGTGCTT

661  CACCACCTGCACCCGACGGTGCCTTGGTGGCGCCTGCCAGCACCCGCACCAAGGGGGAC
   -----+-----+-----+-----+-----+-----+ 720
   GTGGTGGACGTGGGCTGCCACGAACCACCGCGACGGTCTGGGCGTGGTTCCCCCTG

721  ACCGCATGA
   ----- 729
   TGGCGTACT

```

Fig. 32

1 MSAHALPKAD LTATSLIVSG GIIAAWLALH VHALWFLDAA AHPILAVANF  
51 LGLTWLSVGL FIIAHDAMHG SVVPGRPRAN AAMGQLVLWL YAGFSWRKMI  
101 VKHMAHHRHA GTDDDPDFDH GGPVRWYARF IGTYFGWREG LLLPVIVTVY  
151 ALMLGDRWMY VVFWPLPSIL ASIQLFVFGI WLPHRPGHDA FPD RHNARSS  
201 RISDPVSLLT CFHFGGYHHE BHLPTVPWW RLPSTRTKGD TA\*

Fig. 33

```

1  ATGACCAATTTCTGATCGTCGTCGCCACCGTGCTGGTGATGGAGCTGACGGCCTATTCC
   -----+-----+-----+-----+-----+-----+
61  TACTGGTTAAAGGACTAGCAGCAGCGGTGGCAGCACCCTACCTCGACTGCCGATAAGG
   -----+-----+-----+-----+-----+-----+
   GTCCACCGCTGGATCATGCACGGCCCCCTTGGGCTGGGGCTGGCACAAGTCCCACCACGAG
121 -----+-----+-----+-----+-----+-----+
   CAGGTGGCGACCTAGTACGTGCCGGGAACCCGACCCGACCGTGTTTCAGGGTGGTGCTC
   -----+-----+-----+-----+-----+-----+
   GAACACGACCACGCGCTGGAAAAGAACGACCTGTACGGCCTGGTCTTTGCGGTGATCGCC
181 -----+-----+-----+-----+-----+-----+
   CTTGTGCTGGTGCGGACCTTTCTTGCTGGACATGCCGGACCAGAAACGCCACTAGCGG
   -----+-----+-----+-----+-----+-----+
   ACGGTGCTGTTACGGTGGGCTGGATCTGGGCACCGGTCCTGTGGTGGATCGCCTTGGGC
241 -----+-----+-----+-----+-----+-----+
   TGCCACGACAAGTGCCACCCGACCTAGACCCGTGGCCAGGACACCACCTAGCGGAACCCG
   -----+-----+-----+-----+-----+-----+
   ATGACCGTCTACGGGCTGATCTATTTCTGCTCCTGCATGACGGGCTGGTGCATCAGCGCTGG
301 -----+-----+-----+-----+-----+-----+
   TACTGGCAGATGCCCGACTAGATAAAGCAGGACGTAAGTCCCGACCGTAGTCGCGACC
   -----+-----+-----+-----+-----+-----+
   CCGTTCCGCTATATCCCTCGCAAGGGCTATGCCAGACGCTGTATCAGGCCACCGCCTG
361 -----+-----+-----+-----+-----+-----+
   GGCAAGGCGATATAGGAGCGTTCCCGATACGGTCTGCGGACATAGTCCGGGTGCGGAC
   -----+-----+-----+-----+-----+-----+
   CACCACGCGGTGAGGGGCGCGACCATTTGCGTCAGCTTCGGCTTCATCTATGCGCCGCG
421 -----+-----+-----+-----+-----+-----+
   GTGGTGCGCCAGCTCCCCGCGCTGGTAACGCAGTCGAAGCCGAAGTAGATACGCGGCGGC
   -----+-----+-----+-----+-----+-----+
   GTCGACAAGCTGAAGCAGGACCTGAAGACGTGCGGCGTGCTGCGGGCCGAGGCGCAGGAG
481 -----+-----+-----+-----+-----+-----+
   CAGCTGTTCGACTTCGTCTGGACTTCTGCAGCCCGCAGACGCCCCGGCTCCGCGTCTCTC
   -----+-----+-----+-----+-----+-----+
   CGCACC
481 ----- 486
   GCGTGC

```

Fig. 34

1 MTNFLIVVAT VLMELTAYS VHRWIMHGPL GWGWHKSHHE EHDHALEKND  
51 LYGLVFAVIA TVLFTVGWIW APVLWWIALG MTVYGLIYFV LHDGLVHQRW  
101 PFRYIPRKG YARRLYQAHRL HHAVEGRDHC VSFGFIYAPP VDKLKQDLKT  
151 SGVLRAEAQE RT

Fig. 35

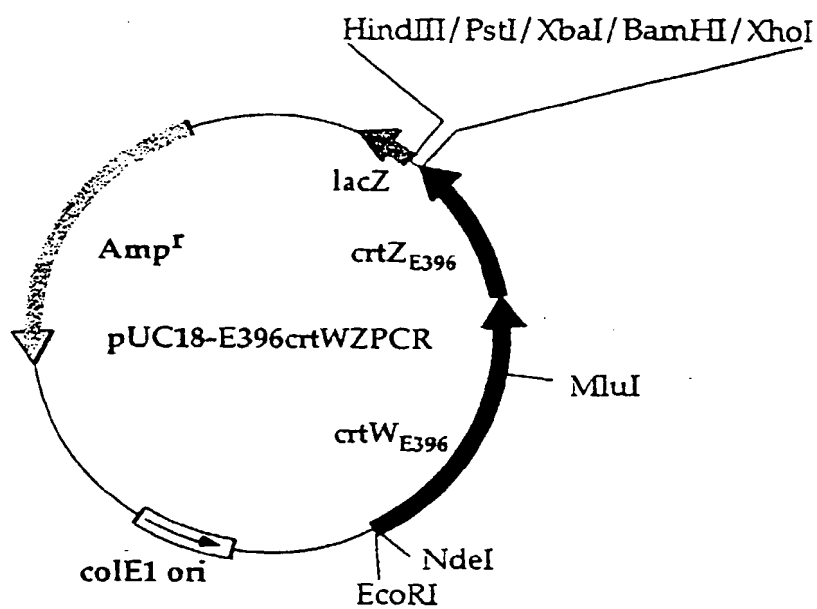


Fig. 36

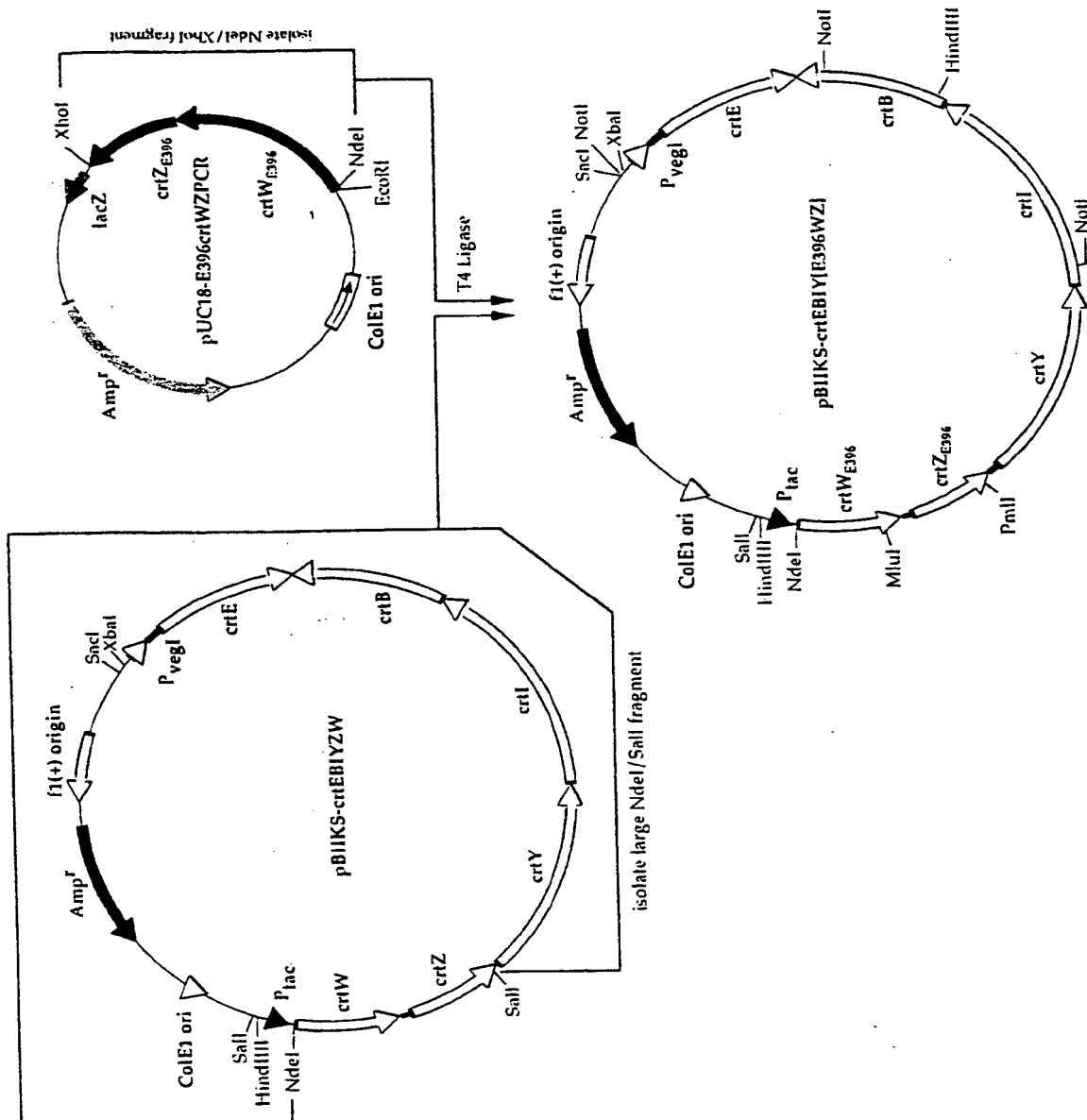


Fig. 37

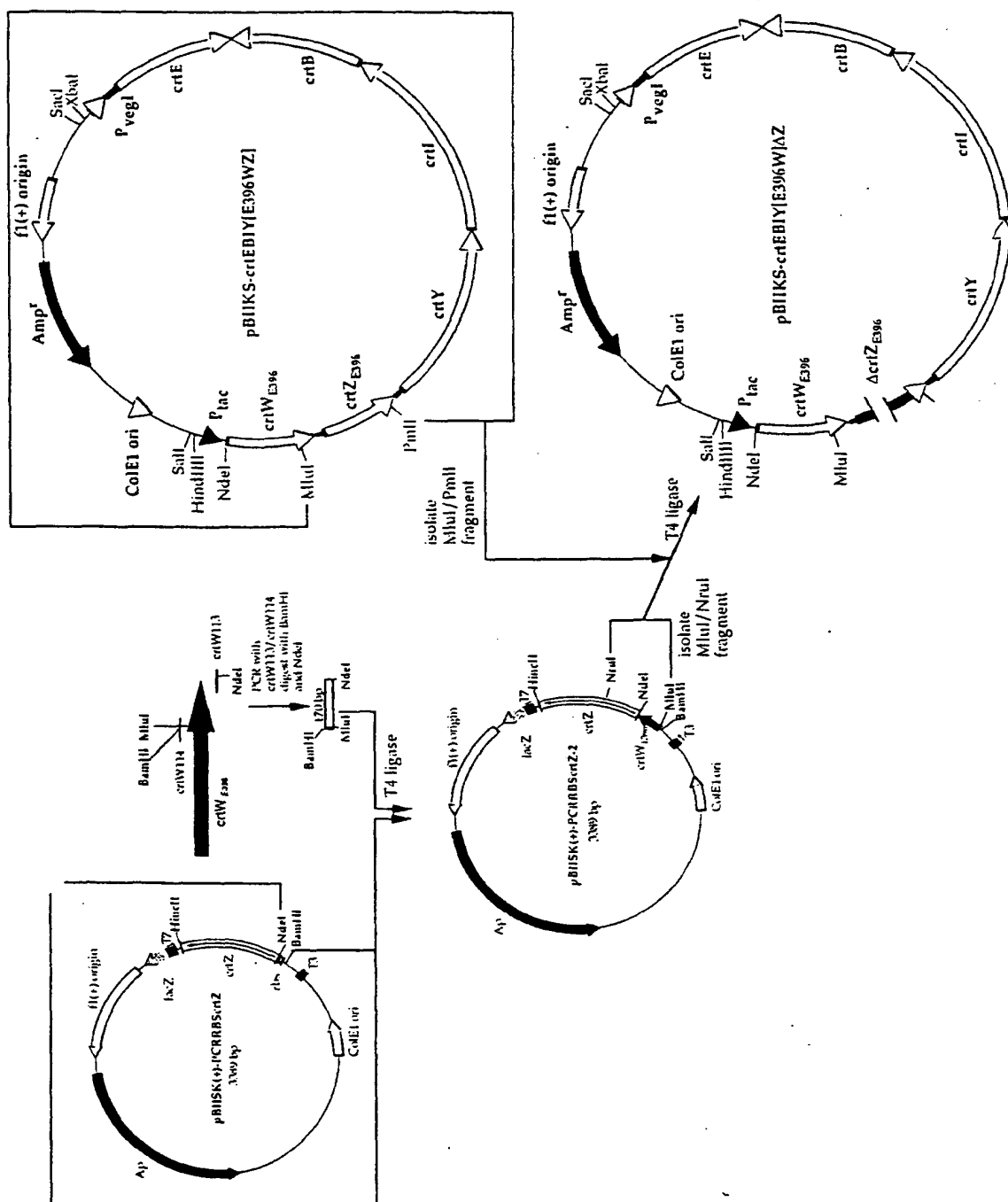


Fig. 38/1

1 CTGCAGGTCTGACACGGCCAGAAGGCCGCGCGCGGGcCGGGGGCCGCcGCATCGCGACC 60  
 -----+-----+-----+-----+-----+  
 GACGTCCAGACTGTGCCGGTCTTCCGGCGCGGCGCCCGCCCCCGGGCGCGTAGCGCTGG  
 61 GGTATCCTTGCCAAGCGCCGCTGGTCGCCCACaACGTCCAGCAGGTCTGCATAGGACTG 120  
 -----+-----+-----+-----+-----+  
 CCATAGGAACGGTTCGCGGCGGACCAGCGGGTGT TGCAGGTCTGCCAGCAGTATCCTGAC  
 121 GAACACCCGGCCCCAGCTGACGGCCAAAGTCGATCATCTGaGTCTGCTCCTCGGCGTCGAA 180  
 -----+-----+-----+-----+-----+  
 CTTGTGGGCCGGGTGCGACTGCCGGTTTCAGCTAGTAGActCAGACGAGGAGCCGCAGCTT  
 181 CTCCTTGATCACGGCCAGCATCTCCAGCCCGGCGATGAACAGCACGCCGGTCTTCAGGTG 240  
 -----+-----+-----+-----+-----+  
 GAGGAAGTAGTGCCGGTCTGAGAGGTGGGCGCGCTACTTGTCTGCGGCCAGAAGTCCAG  
 241 CTGTTCTGTTCGACCCCCGCGCGTCTTTGGOCGCGTGCAGGTCCAGGTCTGGCCGGC 300  
 -----+-----+-----+-----+-----+  
 GACAAGGACAAGCTGGGGGCGCGCAAGAACC GGCGCACGTCCAGGTCCAGGACC GGCCG  
 301 GCACAGGCCCTGCGGCCCCAGGGACCGCGACAGGATCCgcaccagctgcgcccgcaccgt 360  
 -----+-----+-----+-----+-----+  
 CGTGTCCGGGACGCCGGGTCCCTGGCGCTGTCTAGGcgtggtcgacgcgggctggca  
 361 gcccgcgcgcgcgcgcgcaccggccagcagggccatcgccctcggtgatcagggcgatgcc 420  
 -----+-----+-----+-----+-----+  
 cgggctgcgcggcgcggtggccggtcgtcccgtagcggagccactagtcccgctacgg  
 421 gcctagcaccggcgcggtttcgccatgcgccacatgggtcgccggctggccgcggcgcgag 480  
 -----+-----+-----+-----+-----+  
 cggatcgtgccgcgcgaaagcggtacgcggtgtaccagcgcccagccggcgccgcgctc  
 481 cccggcatcgtccatgcagggcaggtcgtcgaagatcagcgatgcggcatgcaccatctc 540  
 -----+-----+-----+-----+-----+  
 gggccgtagcaggtacgtcccgtccagcagcttctagtgcctacgcggtacgtggtagag  
 541 gaccgcgcagggcgcgctcgacgatcgtgtcgacagccccgcccagagcttctgccgcaag 600  
 -----+-----+-----+-----+-----+  
 ctggcgcgctccgcgcgagctgctagcacagcgtctggggcgggctccgaagacggcgctc  
 601 cagcatcagcatgccgcggaacgcttgcccagcagacagcgcccatggctcatggccgg 660  
 -----+-----+-----+-----+-----+  
 gtcgtagtcgtacggcgccctttgcgaacgggctgctgtcgcgcggtaccgagtaccggcc  
 661 gccgagcggtgcgacacggcaccgaatccctgggcgatctcctcaagtctggtctgcag 720  
 -----+-----+-----+-----+-----+  
 cggctcgccgacgctgtgccgtggcttagggaccgctagaggagttcagaccagacgtc  
 721 aaggggtggcgtggatcgggttgacgtctcgtctcatcagtgcccttcgcgcttgggttctg 780  
 -----+-----+-----+-----+-----+  
 ttcccaccgcacctagcccaactgcagagcagagtgtcacggaagcggaaccaagac  
 781 accagggcggaaggtcaggccggggcggcaccccgtagcccgctcatccacgctcaacagt 840  
 -----+-----+-----+-----+-----+  
 tggctccgcccttccagtccggcccccggtggggcactgggcagtaggtggcagttgtca  
 841 ccccatgttggaaggcttcacgcccgatgcgagcccttttcgacggcgacgcggggtcgc 900  
 -----+-----+-----+-----+-----+  
 ggggtacaaccttccgaagtgcgggctaacgctcggaagctgccgctgcgccccagcg  
 901 gcggcaatttntccaacaaggtcagtggaaccggcgcgccgatggccgcgcgcagccaggc 960  
 -----+-----+-----+-----+-----+  
 cgccgttaaanagggtgttccagtcacctggccgcgcggctaccggcgcgcggtcggtccg  
 961 atccttggccggaacaccccgcgccgatcatgatcgccaggtcgtccggcgcgccggc 1020  
 -----+-----+-----+-----+-----+

Fig. 38/2

```

t aggaaccggccttctgtgggcgcgcgcgt agt act agccggctcct agcaggccgcgcgcgcg
1021  gcggcgcaggtcggccgcgcgt caccggattgt caagcaccaggccatcgcgtccgcgcac 1080
-----+-----+-----+-----+-----+
cgccgcgtccagccggcgcgcagtgggccta acagttcgtgggtccggt agcgcaggcgcgtg

ctcgtccgcgcgtcgtccatgtcgacgatcaggccgttctccatgtcgccgaccagttcgcg
1081  -----+-----+-----+-----+-----+ 1140
gagcaggcgcagcaggtacagctgct agtccggcaagaggtacagcgccctggc caagcgc

caccggggcgggtgttcgatcgatcaccaggcatccggtggccatcgccctcggacagggac
1141  -----+-----+-----+-----+-----+ 1200
gtggccccgcacacaagctagct agtggtccgt aggccaccggtagcggagccctgtccctg

caggaggtgacgaagggtccggtgaaatagacatgcgcgtgcgagggcctgcag
1201  -----+-----+-----+-----+-----+ 1253
gtcctccactgcttcccagaccactttatctgtacgcgcacgctccggacgtc

```

Fig. 39

```

1  ATGAGACGAGACGTCAACCCGATCCACGCCACCCTTCTGCAGACCAGACTTGAGGAGATC 60
   -----+-----+-----+-----+-----+
   TACTCTGCTCTGCAGTTGGGCTAGGTGCGGTGGGAAGACGTCTGGTCTGAACCTCTCTAG

61  GCCCAGGGATTTCGGTGCCGTGTCCGAGCCGCTCGGCCCGGCCATGAGCCATGGCGCGCTG 120
   -----+-----+-----+-----+-----+
   CGGGTCCCTAAGCCACGGCACAGCGTCGGCGAGCCGGGCCGGTACTCGGTACCGCGCGAC

121  TCGTCGGGCAAGCGTTTCCGCGGCATGCTGATGCTGCTTGCAGCAGAAGCCTCGGGCGGG 180
   -----+-----+-----+-----+-----+
   AGCAGCCCGTTTCGCAAAGGCGCCGTACGACTACGACGAACGCCGTCTTCGGAGCCCGCCC

181  GTCTGCGACACGATCGTCCAGCGCGCCTGCGCGGTGAGATGGTGCATGCCGCATCGCTG 240
   -----+-----+-----+-----+-----+
   CAGACGCTGTGCTAGCAGCTGCGGCGGACGCGCCAGCTCTACCACGTACGGCGTAGCGAC

241  ATCTTCGACGACCTGCCCTGCATGGACGATGCCGGGCTGCGCCGCGGCCAGCCCGCGACC 300
   -----+-----+-----+-----+-----+
   TAGAAGCTGCTGGACGGGACGTACCTGCTACGGCCCGACGCGGCGCCGGTTCGGGCGCTGG

301  CATGTGGCGCATGGCGAAAGCCGCGCCGTGCTAGGCGGCATCGCCCTGATCACCGAGGCG 360
   -----+-----+-----+-----+-----+
   GTACACCGCGTACCGCTTTCGGGCGCGGCACGATCCGCCGTAGCGGGACTAGTGGCTCCGC

361  ATGGCCCTGCTGGCCGGTGCAGCGCGGCGCGCTCGGGCACGGTGCAGGCGCAGCTGGTGCAG 420
   -----+-----+-----+-----+-----+
   TACCGGGACGACCGGCCACGCGCGCCGCGCAGCCCGTGCCACGCCCGCGTTCGACCACGCC

421  ATCTGTGCGCGTCCCTGGGGCCGAGGGCCTGTGCGCCGGCCAGGACCTGGACCTGCAC 480
   -----+-----+-----+-----+-----+
   TAGGACAGCGCCAGGGACCCCGCGTCCCGGACACGCGGCCGCTCCTGGACCTGGACGTG

481  GCGGCCAAGAACGGCGCGGGGTGGAACAGGAACAGGACCTGAAGACCGCGTGCTGTTC 540
   -----+-----+-----+-----+-----+
   CGCCGGTTCTTGCCGCGCCCCAGCTTGTCTTGTCTGGACTTCTGGCCGCACGACAAG

541  ATCGCCGGGCTGGAGATGCTGGCCGTGATCAAGGAGTTCGACGCCGAGGAGCAGACTCAG 600
   -----+-----+-----+-----+-----+
   TAGCGGCCCGACCTCTACGACCGGCACTAGTTCTTCAAGCTGCGGCTCCTCGTCTGAGTC

601  ATGATCGACTTTGGCCGTAGCTGGGCCGGGTGTTCCAGTCTATGACGACCTGCTGGAC 660
   -----+-----+-----+-----+-----+
   TACTAGCTGAAACCGGCAGTCGACCCGGCCACAAGGTCAGGATACTGCTGGACGACCTG

661  GTTGTGGCGACACAGGCGCGCTTGGCAAGGATACCGGTGCGGATGCGGCGGCCCCCGGC 720
   -----+-----+-----+-----+-----+
   CAACACCCGCTGGTCCGCGCGGAACCGTTCCTATGGCCAGCGCTACGCCGCGGGGGCGG

721  CCGCGCGCGCGCCTTCTGGCCGTGTGACACCTGCAGAACGTGTCCCGTCACTATGAGGCC 780
   -----+-----+-----+-----+-----+
   GGGCGCGCGCGGAAGACCGGCACAGTCTGGACGTCTTGACAGGGCAGTGATACTCCGG

781  AGCCGCGCCCAGCTGGACGCGATGCTGCGCAGCAAGCGCCTTCAGGCTCCGGAATCGCG 840
   -----+-----+-----+-----+-----+
   TCGGCGCGGGTTCGACCTGCGCTACGACGCGTCTTCGCGGAAGTCCGAGGCCTTAGCGC

841  GCCCTGCTGGAACGGGTTCGCGCTACGCGCGCGCGCGCTAG 882
   -----+-----+-----+-----+
   CGGGACGACCTTGCCCAAGACGGGATGCGGCGCGCGCGGATC

```

Fig. 40

1 MRRDVNPIHA TLLQTRLEEI AQGFGAVSQP LGPAMSHGAL SSGKRFRGML  
51 MLLAAEASGG VCDTIVDAAC AVEMVHAASL IFDDLPCMDD AGLRRGQPAT  
101 HVAHGESRAV LGGIALITEA MALLAGARGA SGTVRAQLVR ILSRSLGPQG  
151 LCAGQDLDLH AAKNGAGVEQ EQDLKTGVLF IAGLEMLAVI KEFDAEEQTQ  
201 MIDFGRQLGR VFQSYDDLDD VVGDAQALGK DTGRDAAAPG PRRGLLAVSD  
251 LQNVSRHYEA SRAQLDAMLR SKRLQAPEIA ALLERVLPYA ARA\*

Fig. 41

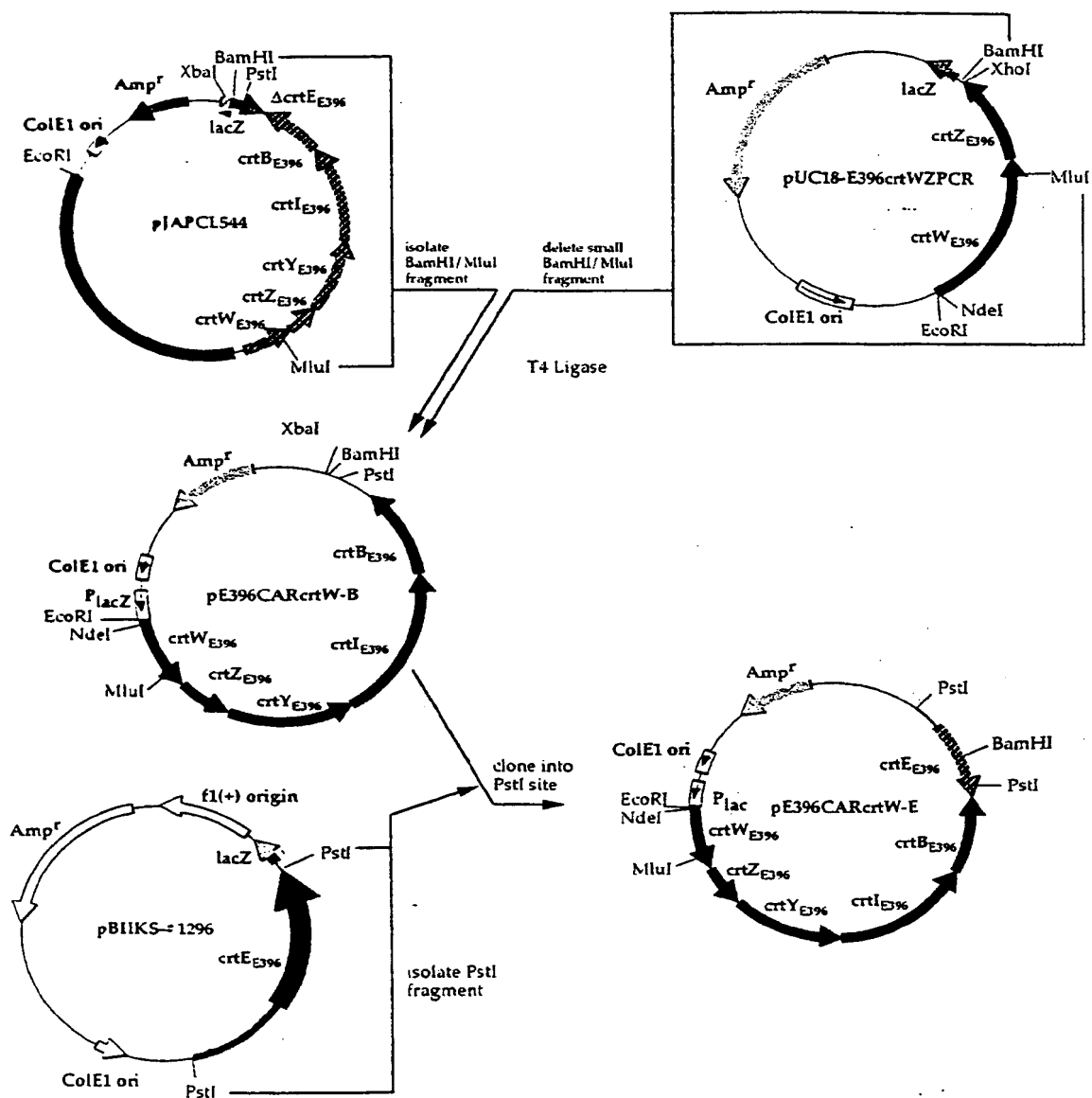


Fig. 42

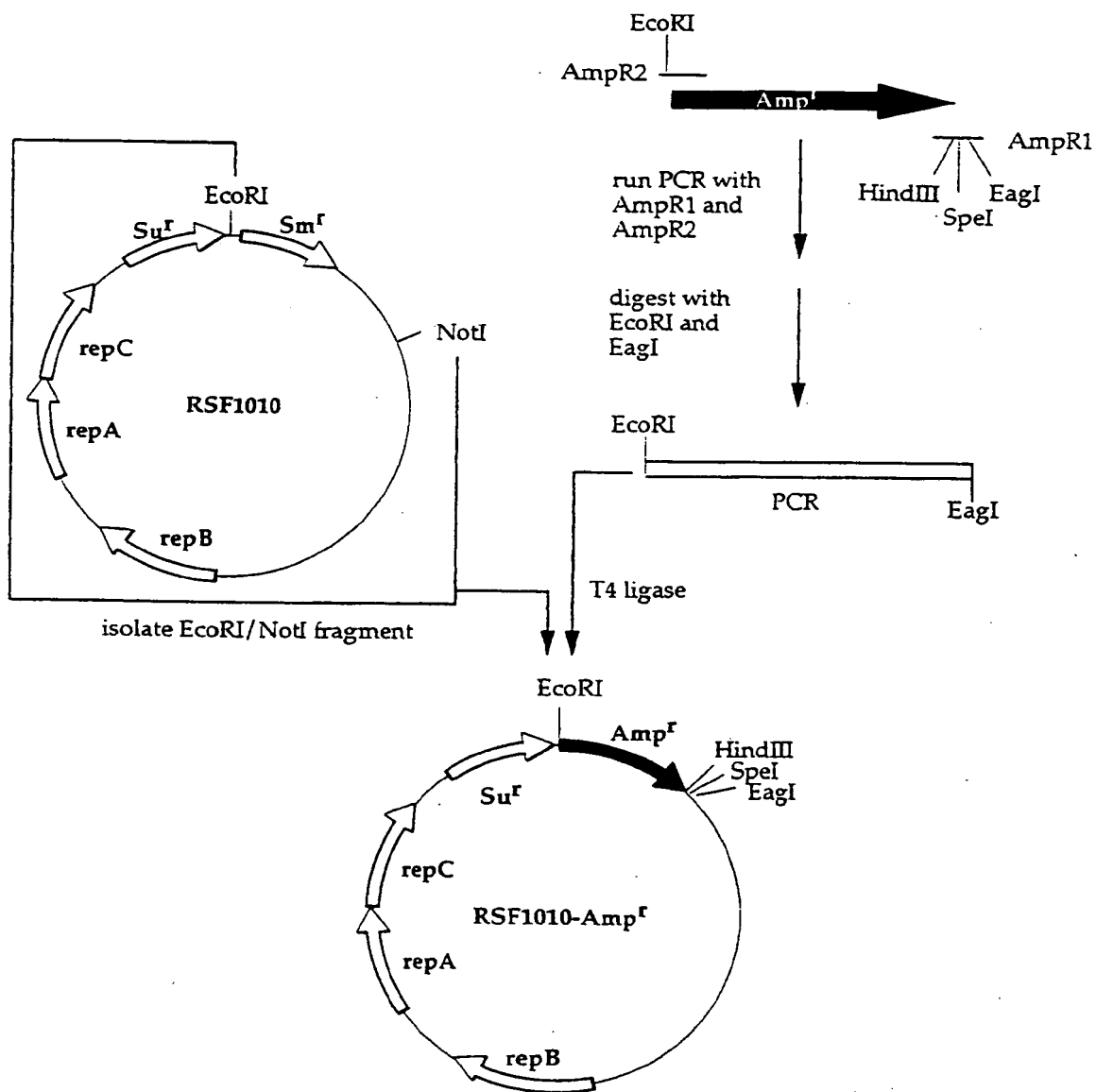
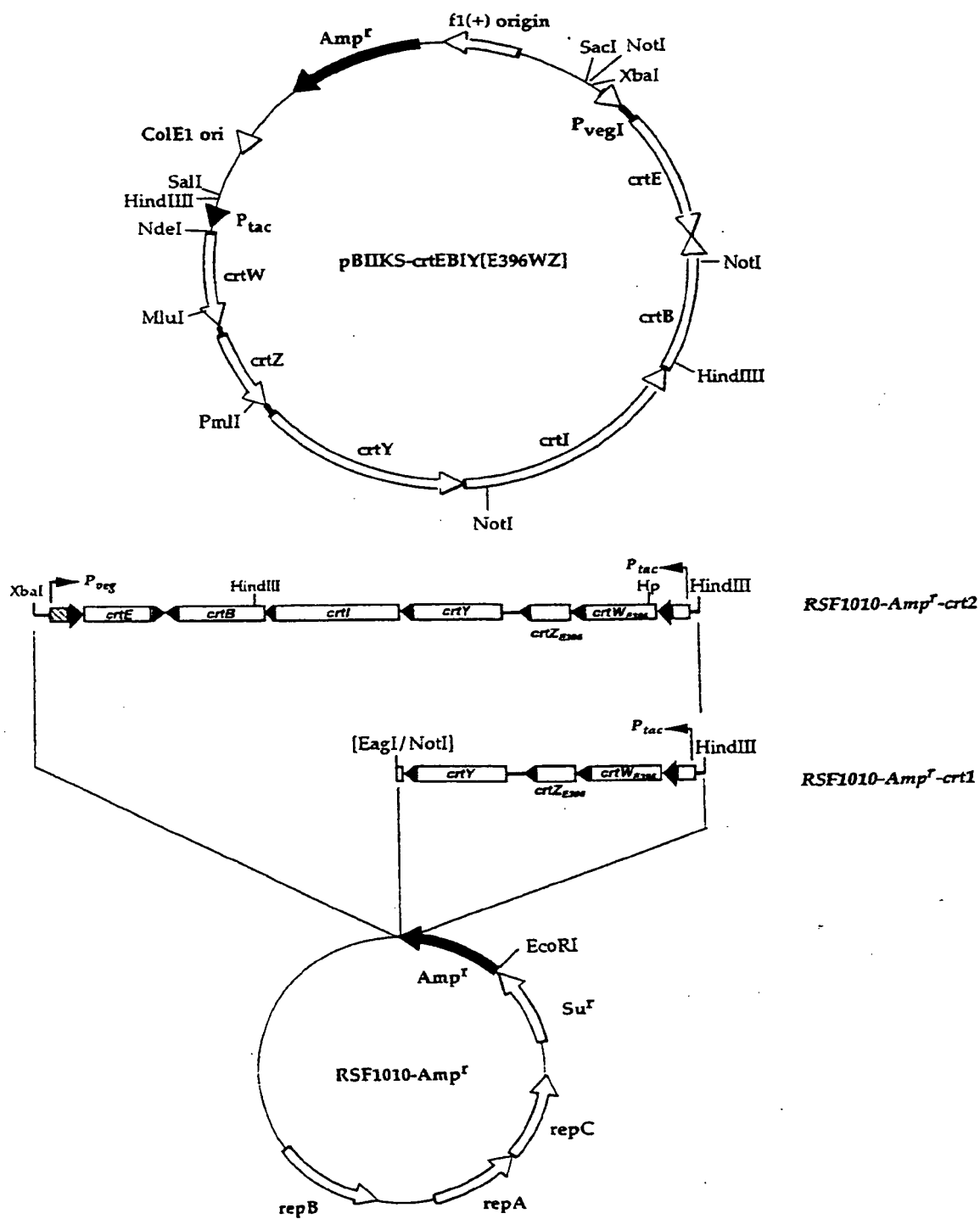
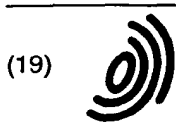


Fig. 43





Europäisches Patentamt

European Patent Office

Office européen des brevets



(11) **EP 0 872 554 A3**

(12) **EUROPEAN PATENT APPLICATION**

(88) Date of publication A3:  
07.06.2000 Bulletin 2000/23

(43) Date of publication A2:  
21.10.1998 Bulletin 1998/43

(21) Application number: 97120324.5

(22) Date of filing: 20.11.1997

(51) Int. Cl.<sup>7</sup>: **C12N 15/52**, C12P 23/00,  
A23K 1/16, A23L 1/275,  
C12N 1/21

(84) Designated Contracting States:  
**AT BE CH DE DK ES FI FR GB GR IE IT LI LU MC  
NL PT SE**  
Designated Extension States:  
**AL LT LV MK RO SI**

(30) Priority: 02.12.1996 EP 96810839

(71) Applicant:  
**F. HOFFMANN-LA ROCHE AG  
4070 Basel (CH)**

(72) Inventors:  
• **Pasamontes, Luis**  
4632 Trimbach (CH)  
• **Tsygankov, Yuri**,  
State Scient. Cnt. of Russian Fed  
Moscow 113545 (RU)

(74) Representative: **Braun, Axel et al**  
**F.Hoffmann-La Roche AG**  
**Patent Department (PLP),**  
**124 Grenzacherstrasse**  
**4070 Basel (CH)**

(54) **Improved fermentative carotenoid production**

(57) The present invention is directed to processes for the preparation of canthaxanthin, adonixanthin, astaxanthin, a mixture of adonixanthin and astaxanthin and zeaxanthin by a cell which has been transformed by DNA sequences encoding the respective biosynthetic enzymes of Flavobacterium and the gram negative bacterium E-396. Furthermore the present invention is directed to a food or feed composition comprising one or more of the aforementioned carotenoids.

**EP 0 872 554 A3**



European Patent  
Office

# EUROPEAN SEARCH REPORT

Application Number  
EP 97 12 0324

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
D,A	EP 0 735 137 A (KIRIN BREWERY ;MARINE BIOTECH INST CO LTD (JP)) 2 October 1996 (1996-10-02) * page 5, line 55 - page 7, line 32 * * page 10 - page 11 * * page 14 - page 21; examples 7,8,12,13 * ---	1-7	C12N15/52 C12P23/00 A23K1/16 A23L1/275 C12N1/21
A	EP 0 635 576 A (NIPPON OIL CO LTD) 25 January 1995 (1995-01-25) * examples * ---	1-7	
D,A	WO 91 13078 A (AMOCO CORP) 5 September 1991 (1991-09-05) * page 24 - page 43 * * page 55 - page 64 * * page 164 - page 176; examples 21-24 * ---	3,5-7	
P,X	EP 0 747 483 A (HOFFMANN LA ROCHE) 11 December 1996 (1996-12-11) * the whole document * ---	2,3,5-7	
P,X	PASAMONTES L ET AL: "Isolation and characterization of the carotenoid biosynthesis genes of Flavobacterium sp. strain R1534" GENE: AN INTERNATIONAL JOURNAL ON GENES AND GENOMES, vol. 185, no. 1, 31 January 1997 (1997-01-31), pages 35-41, XP004093151 ISSN: 0378-1119 * the whole document * -----	3,6	TECHNICAL FIELDS SEARCHED (Int.Cl.6)  C12N C12P
The present search report has been drawn up for all claims			
Place of search <b>THE HAGUE</b>		Date of completion of the search <b>19 April 2000</b>	Examiner <b>Andres, S</b>
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document			

EPO FORM 1503 03/82 (P/MC01)

**ANNEX TO THE EUROPEAN SEARCH REPORT  
ON EUROPEAN PATENT APPLICATION NO.**

EP 97 12 0324

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on  
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

19-04-2000

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0735137 A	02-10-1996	AU 702584 B	25-02-1999
		AU 1281195 A	17-07-1995
		FI 962633 A	23-08-1996
		NO 962689 A	27-08-1996
		US 5811273 A	22-09-1998
		CA 2180024 A	06-07-1995
		WO 9518220 A	06-07-1995
		US 5972690 A	26-10-1999
EP 0635576 A	25-01-1995	CA 2128549 A	23-01-1995
		JP 7079796 A	28-03-1995
		NO 942731 A	23-01-1995
		US 5607839 A	04-03-1997
		US 5858761 A	12-01-1999
WO 9113078 A	05-09-1991	CA 2055447 A	03-09-1991
		EP 0471056 A	19-02-1992
		JP 5504686 T	22-07-1993
		US 5545816 A	13-08-1996
		US 5530188 A	25-06-1996
		US 5530189 A	25-06-1996
		US 5684238 A	04-11-1997
		US 5618988 A	08-04-1997
		US 5656472 A	12-08-1997
EP 0747483 A	11-12-1996	JP 9023888 A	28-01-1997

EPO FORM P0459

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

